

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K	A2	(11) International Publication Number: WO 97/10796 (43) International Publication Date: 27 March 1997 (27.03.97)
<p>(21) International Application Number: PCT/IB96/01021</p> <p>(22) International Filing Date: 30 August 1996 (30.08.96)</p> <p>(30) Priority Data: 60/003,114 1 September 1995 (01.09.95) US</p> <p>(71) Applicant (for all designated States except US): RAMOT-UNIVERSITY AUTHORITY FOR APPLIED RESEARCH AND INDUSTRIAL DEVELOPMENT LTD. [IL/IL]; 32 Haim Levanon Street, Ramat-Aviv, 61392 Tel-Aviv (IL).</p> <p>(72) Inventor; and (75) Inventor/Applicant (for US only): LAVI, Sara [IL/IL]; 6 Tnuat Hameri Street, 55000 Kyriat Ono (IL).</p> <p>(74) Agent: HACKMEY, Michal; A.E. Mulford, 11 Shlom zion Hamalka Street, P.O. Box 544, 91004 Jerusalem (IL).</p>		<p>(81) Designated States: AL, AU, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KP, KR, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>Without international search report and to be republished upon receipt of that report.</i></p>
<p>(54) Title: MANIPULATION AND DETECTION OF PROTEIN PHOSPHATASE 2C - PP2Cα - EXPRESSION IN TUMOR CELLS FOR CANCER THERAPY, PREVENTION AND DETECTION</p>		
<p>(57) Abstract</p> <p>A method of detecting cancer in a patient by detecting alteration of activity of the gene coding for human type protein phosphatase 2C (PP2Cα and PP2Cβ) and genetic polymorphisms thereof in a specimen isolated from the patient is disclosed. The invention further provides a method of treating cancer including the steps of first determining the type of cancer and cells expressing the cancer and then preparing a vector which will specifically target the cancer cells and can include regulatory elements to control the expressibility of PP2Cα. The vector is then administered to the patient. Alternatively an antisense vector can be prepared.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

-1-

MANIPULATION AND DETECTION OF PROTEIN
PHOSPHATASE 2C - PP2C α - EXPRESSION IN TUMOR
CELLS FOR CANCER THERAPY, PREVENTION AND
DETECTION

5

BACKGROUND OF THE INVENTION

TECHNICAL FIELD

10 The present invention relates to detection and
methods of treating cancer by utilizing the gene human
type protein phosphatase 2C (PP2C α and PP2C β) and gene
products thereof and kits for the practice of the
invention; preparing native and transgenic organisms in
15 which the gene products encoded by the human PP2C α gene
or its homolog in other species are produced, or the
expression of the native PP2C α gene is modified or
knocked out.

20

BACKGROUND ART

Transformed or malignant cells pose a severe
health threat. If the transformed phenotype can be
reversed the cancerous cells can be controlled
providing a treatment. In reversal the cells can loose
25 their neoplastic phenotype thereby reinstating normal
cellular growth and/or differentiation, or there is
growth arrest or there can be activation of programmed
cell death - apoptosis pathway. It would be useful to
provide therapeutic measures which can reverse the
30 transformed phenotype by instituting any of these
reversal means by therapeutic measures. Further, early
identification of transformation events would also
prove useful, since therapy could be initiated sooner.

Genes have now been identified that are involved
35 in transformation such as Ras, Fos PDGF, erb-B, erb-B2,
RET, c-myc, Bcl-2, APC, NF-1, RB, p53, etc. The genes

-2-

fall into two broad categories proto-oncogenes and tumor suppressor genes. Proto-oncogenes code for proteins that stimulate cell division and when mutated (oncogenes) cause stimulatory proteins to be overactive
5 with the result that cells over proliferate. Tumor suppressor genes code for proteins that suppress cell division. Mutations and/or aberrant regulation can cause these proteins to be inactivated thereby rendering the cells without proliferation restraint.
10 Additionally, E2F and p53 and others can act as both oncogene and tumor suppressor gene when improperly expressed. Among the oncogenes and tumor suppressor genes are motifs which act as transcription factors and as protein kinase. The identification of these
15 specific genes have disclosed some of how the cell life cycle progresses.

Gene amplification is one of the distinct abnormalities associated with malignant cells and transformed cell lines [see generally "Gene
20 Amplification in Mammalian Cells, A comprehensive Guide. edited by R. E. Hellems, Marcel Dekker, Inc. for a review of amplification.] This phenomenon is part of the genetic instability characterizing neoplastic cells and occurs rarely in normal cells. Some oncogenes and
25 tumor suppressor genes have been shown to be amplified such as Ras, Erb, p53 etc.

Phosphorylation of structural and regulatory proteins including oncogenes and tumor suppressor genes is a major intracellular control mechanism in
30 eukaryotes [Wera and Hemmings, 1995; Cohen, 1989]. Protein phosphorylation and dephosphorylation is part of the regulatory cycle for signal transduction, cell cycle progression and transcriptional control. Protein kinases and protein phosphatases both have roles in the
35 phosphorylation - dephosphorylation cycle, respectively. Mutations in the genes coding for these

proteins can lead to failure of protein phosphorylation. For example, in yeast, mutations of a type 2C protein phosphatases lead to a defect in osmoregulation [Shiozaki and Russell, 1995].

5 pp2c is a protein serine/threonine phosphatase [Cohen 1989]. The pp2c family consists of two cytoplasmic isoenzymes in mammalian tissues [McGowan and Cohen, 1987] and at least three pp2c-like enzymes in yeast show the same enzymatic and biochemical
10 properties. The two mammalian isoenzymes are monomers but differ slightly in molecular mass (44KDa and 42KDa) and are designated pp2c α and pp2c β . There is conflicting literature as to their function and association of these protein phosphatases with
15 transformed cells [Saadat et al, 1994; Nishikawa, et al, 1995; Lau and Baylink, 1993; Shiozaki et al, 1994; Eden and Cedar, 1994; McGowan and Cohen, 1987; Wenk and Mieskes, 1995]

20 It would be useful to be able to therapeutically control protein phosphorylation where needed for normal cell function. Additionally, glycosylation following mRNA translation is essential for the functioning of many gene products. Aberrant glycosylation of proteins can interfere with protein function and can result from
25 an altered regulatory pathway. An important mode of control of gene expression is DNA methylation [Eden and Cedar, 1994]. Aberrant methylation of DNA can play a role and lead to improper expression of regulatory proteins controlling cell cycle.

30 Viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Adeno associated viruses are members of the family of parvoviruses for which tumor suppressive properties have already been described in 1960 [for
35 review see Rommelaere and Tattersal, 1990]. They are a group of small viruses, with a ssDNA genome of

approximately 5000 nucleotides, characterized by identical palindromic termini of 154 bases. The left part of the AAVDA3 genome encodes four multifunctional, overlapping, non-structural proteins (Rep78, Rep68, Rep52 and Rep40) which are translated from differentially spliced mRNA driven by the P5 and P19 promoters (Accession numbers J01901, M12405, M12468, M12469). In the right part of the genome three overlapping capsid polypeptides (VP1 - VP3) are encoded from the P40 promoter [Berns, 1990; Leonard and Berns, 1994]. These extremely small DNA viruses are represented in vertebrates by two genera, the autonomously replicating and the helper dependent parvovirus [Siegl et al., 1985].

The helper-dependent adeno-associated viruses (AAV) depend for their replication on coinfecting helper virus [Young and Mayor, 1979a,b], or on conditions of genotoxic stress [Yakobson et al., 1987] and comprise agents infecting humans without apparent disease [Cukor et al., 1984]. Helper viruses are adenoviruses [Atchison et al., 1965], herpes group viruses [Salo and Mayor, 1979] and vaccinia virus [Schlehofer et al., 1986]. The helper viruses share the ability to induce chromosomal damage early in their infection cycle [Schlehofer and zur Hausen, 1982].

Tumor suppressive properties have been found for AAV [for review see Schlehofer, 1994]. It has been shown that the development of tumors induced in rodents by adenoviruses, herpes viruses or by transplantation of cells transformed by these viruses could be inhibited by infecting the animal cells with AAV [Kirschstein et al. 1968; Mayor et al., 1973; de la Maza and Carter, 1981; Ostrove et al., 1981]. The *in vivo* findings of tumor suppression are paralleled by results showing inhibition of cellular transformation *in vitro*. This could be shown for cells of different origin

(hamster and mouse) transformed by viruses or by activated oncogenes. Compared with controls, cells infected with AAV or transfected with specific AAV DNA sequences displayed decreased focus formation and saturation density indicating inhibition of transformation-associated traits [Casto and Goodheart, 1972; Katz and Carter, 1986; Hermonat, 1989; Hermonat, 1994; Schlehofer et al, 1983; Schlehofer, 1994; Yang et al, 1995; Kleinschmidt et al, 1995].

In addition, there are seroepidemiologic findings in the human population, showing that cancer patients exhibit antibodies to AAV less frequently than matched control individuals. Three independent studies carried out in the USA [Mayor et al., 1976], Belgium [Sprecher-Goldberger et al., 1971] and Germany [Georg-Fries et al., 1984] using different serologic techniques, have found a high prevalence of antibodies to AAV (types 2, 3, and 5) in the normal population contrasting with a relatively low frequency of seropositivity in patients with cancer.

It would be useful to develop therapeutic methods for controlling cell transformation. As the above information indicates, it is possible to reverse cell transformation or to specifically kill the transformed cell. Given the available anti-sense technology, vector delivery technology and the like it would be useful to find human cellular mechanisms that can be controlled to reverse cell transformation with these methods or others as they become known.

SUMMARY OF THE INVENTION

According to the present invention, a method and kit of detecting cancer in a patient by detecting alterations of the activity of the gene (PP2C α or PP2C β) coding for human type protein phosphatase 2C

-6-

(pp2c α) and genetic polymorphisms thereof in a specimen isolated from the patient is disclosed.

The invention further provides a method of treating cancer including the steps of first
5 determining the type of cancer and cells expressing the cancer and then preparing a vector which will specifically target the cancer cells and can include regulatory elements to control the expressibility of PP2C α . The vector is then administered to the patient.
10 Alternatively an antisense vector can be prepared.

The invention further provides a method of treating diseases due to aberrant phosphorylation due to alteration of expression of PP2C α by controlling PP2C α expression.

15

BRIEF DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better
20 understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIGURE 1A-B are graphs of a FACS analysis of CO60 and two AAV/neo cell lines 913 and 916 as prepared for
25 cell cycle analysis. 24 hours after seeding the cell were trypsinized and washed with PBS. The cells were resuspended in 1ml buffer containing 0.1% sodium citrate, 0.1% triton X-100 and 50 μ g propidium iodide, and then processed in the FACS.

FIGURE 2 is a photograph of a Southern Blot
30 Analysis showing CHINT is associated with AAV integration in different AAV/neo cell lines. Southern blot analysis of different AAV/neo clones, CO60 DNA, digested with *Bgl*II, and hybridized with "CHINT" probe.
35 9-1, 2, 3, 4 and 5 on AAV/neo cell lines. 93R is a

-7-

revertant that lost the whole chromosome containing the AAV. A6 is a mouse cell line.

FIGURE 3 is a schematic representation of the organization of the integrated AAV and the flanking cellular sequences in 9-3 cells. A genomic library was prepared from C9-3 cells using the EMBL-4 lambda phage and scored for AAV positive clones. A clone of 13Kb-λSL9-1 was isolated and later subcloned to a blue-script vector. Plasmids pSL9-11 (13Kb), pSL9-8 (10Kb) and pSL9-6 (3Kb) were obtained as indicated in the figure.

FIGURE 4A-B wherein (A) is a photograph of a Southern Blot Analysis showing AAV is adjacent to the gene coding to PP2Cα in 9-3 cells. The Southern blot analysis of genomic DNA, from CO60 and 9-3 cells digested by *EcoRI*, or *XbaI* was hybridized sequentially with the following probes: 1) AAV; 2) CHINT; and 3) Rat PP2Cα probes. The CHINT and the PP2Cα sequences are adjacent (4Kb *EcoRI* fragment). The AAV CHINT and PP2Cα are in a close proximity in 9-3 cells (the 5.6Kb *XbaI* fragment). (B) pSL9-6 is adjacent to PP2Cα in the wild type Chinese hamster cells. *BamHI* digested DNA from the Chinese hamster neo cells was hybridized to pSL9-6 and PP2Cα probes. A common fragment of ~8.5Kb appeared in all cell lines including CO60. The same fragment hybridized also to CHINT probe (data not shown).

FIGURE 5A-C are photographs of a Southern blot analysis of DA3 (lane 8) and DA3J1-DA3J7 cells lines (lanes 1-7). Genomic DNA was digested with *BglII*. The blots were hybridized sequentially with an AAV/neo JDT277, pSL9-6 and PP2Cα PCR probes. A 4Kb fragment hybridized to the AAV probe and pSL9-6 probe in J3 (lane 3), J4 (lane 4) and J6 (lane 6). A fragment smaller than 4Kb hybridized to both AAV and PP2Cα probe in J1 (lane 1), J2 (lane 2), J5 (lane 5) and J6 (lane 6).

FIGURE 6 is a photograph which shows the alteration in PP2C α mRNA in response to carcinogen treatment. Forty μ g of total RNA were isolated from CO60 and C9-3 cells 48 hours after treatment with MNNG (7.5 μ g/ml and 2.5 μ g/ml respectively), and from untreated cells and fractionated on a denaturing gel (1.2% agarose/6.6% formaldehyde gel). The gel was blotted and hybridized consecutively with 32 P-labeled rat PP2C α cDNA (A) pSL9-1^{DNA} (B) and rRNA cDNA (C).

FIGURE 7 is a photograph which shows gel electrophoresis analysis after 25, 30 and 35 PCR cycles. The oligo dT-primed cDNA obtained from colorectal tumor No. 6 (T6), or from its adjacent nontumorous mucosa (N6), were subjected to PCR reactions using the specific PP2C α and the β -actin sense and anti-sense primers. The 25 cycle PCR cycle for PP2C α cDNA in the normal and tumor tissues is not shown since a visible product was not found.

FIGURE 8 is a photograph which shows gel electrophoresis analysis after 25, 30, 30 and 35 PCR cycles of aliquots of the oligo dT-primed cDNA obtained from CHE cell line, or from its adjacent transformed cell line (CO60), subjected to PCR reactions using the specific PP2C α and the β -actin sense and anti-sense primers.

FIGURE 9A-B are schematic representations of plasmids that contain PP2C α cDNA in the (A) sense orientation (pYM001) and in the (B) antisense orientation (pYM002).

FIGURE 10 is a photograph which shows gel electrophoresis analysis of immunoprecipitation of liver extracts with a panel of monoclonal antibodies raised against pp2c α ; 1D5, 2A3, 9F4, 9F1, are monoclonal antibodies used to precipitate pp2c α from liver extract; 801 and 351 are rabbit polyclonal antibodies used for detection after immunoblotting.

FIGURE 11 is a schematic representation of a genomic λ 100 clone containing the first translated exon of PP2C α . The phage was cloned from a CHO library. The sequenced regions are indicated by cross hatching
5 (SEQ ID Nos:15 and 16).

FIGURE 12 is a photograph which shows gel electrophoresis wherein lane 1: Cotransfection with pSK1 and pAV2; lane 2: Transfection with the SV40 plasmid pSK1 SV40 replicates; lane 3: Cotransfection of
10 pSVK1 and a plasmid harboring 140bp from the AAV genome nucleotide 125-263; lane 4: Cotransfection of pSVK1 with pSL9-6.

FIGURE 13 is a photograph of a Northern blot wherein RNA from various mouse tissues is hybridized
15 with PP2C α cDNA demonstrating that there are several mRNAs of different sizes ranging from less than 2 kb to higher than 5.0kb. RNA was extracted from ovary (O), Testis (T), Kidney (K), Liver (L), Muscle (M), Heart (H), Lung (Lu) and Brain (B).

20

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention discloses a method of
25 detecting cancer in a patient by detecting alterations in gene activity of the gene (PP2C α) coding for human type protein phosphatase 2C (pp2c α) and genetic polymorphisms thereof in a specimen isolated from the patient. The gene activity of the patient is compared
30 to that of normal controls. Alterations in activity can be a down-regulation of the gene activity or conversely an up-regulation resulting in changes in phosphorylation. Further, alterations can result in aberrant function or absence of the gene product and in
35 a change in distribution of the gene product within the cell itself.

-10-

Polymorphisms are variants in the gene sequence. They can be sequence shifts found between different ethnic and geographic locations which, while having a different sequence, produce functionally equivalent gene products, isoforms. Polymorphisms also encompass variations which can be classified as alleles and/or mutations which can produce gene products which may have an altered function. Polymorphisms also encompass variations which can be classified as alleles and/or mutations which either produce no gene product, an inactive gene product or increased levels of gene product. Polymorphisms as used herein can also encompass variations which are due to differences in DNA methylation in control and coding regions. Further, the term is also used interchangeably with allele as appropriate.

Cancer is defined as transformed or malignant cells, i.e. cells undergoing uncontrolled growth and spread (see generally, Scientific American September, 1996 for a review).

In general, it is found in cell transformation as shown in the Examples herein below (Examples 4, 5) that the activity/expression of PP2C α is reduced compared to that of normal controls as determined by a reduction in the amount of gene product in the cell. However, it is contemplated by the present invention that increased activity results in changes in protein activity such that there are changes in cell function.

Further it is recognized by the present invention that there is more than one form of the gene product of PP2C α and that one may be reduced or altered in cells while another specific form of PP2C α will be elevated or more prominent compared to normal controls. The cells can be any cell type that shows alteration in PP2C α activity in a disease state. Further, a second gene may be controlled by the alteration in the

-11-

activity of PP2C α such that their products are elevated or reduced and can be monitored by the method of the present invention. New transcripts, absence of transcripts or alterations in the protein coded by these transcripts are monitored.

Further, the present invention recognizes that pp2c α is itself phosphorylated as it has several phosphorylation sites including tyrosine, serine and threonine and that failure to phosphorylate it properly will cause malfunction of the pp2c α protein. Further, pp2c α also dephosphorylates itself. A failure in its autophosphorylation will have effects on cell cycle regulation.

Samples can be biopsied material from suspected precancerous lesions or any tissue or bodily fluid which can be assayed for PP2C α activity or gene product as described herein. Bodily fluids such as blood, urine, cerebrospinal fluid and saliva can be examined as is appropriate.

In an embodiment the detection of PP2C α activity is by assaying the specimen for mRNA complementary to PP2C α DNA including polymorphisms thereof with an assay selected from the group consisting of *in situ* hybridization, Northern blotting and reverse transcriptase - polymerase chain reaction.

In an alternative method, the detecting of PP2C α activity and cellular distribution is by assaying the specimen for a PP2C α gene product including polymorphisms and peptide fragments thereof with an assay selected from the group consisting immunohistochemical and immunocytochemical staining, ELISA, RIA, immunoblots, immunoprecipitation, Western blotting, functional assays for activity of gene product, assays for phosphorylation patterns and protein truncation test. Target proteins which are dephosphorylated by pp2c α can have different size

characteristics on PAGE and different isoelectric points as well as changes in function such as their ability to interact with other proteins, RNA, DNA and other cellular components.

5 Further, if chromosomal abnormalities are associated with altered PP2C α these are screened for using standard methods known in the art.

In addition to changes in the location and amount of the gene product in the cell itself as shown in the
10 Examples herein below, the method of the present invention screens for the gene product in bodily fluids. With alteration in gene function the level of gene product in the bodily fluid is affected as for example more can be released from the cell if
15 glycosylation or signal sequences are affected. Incomplete protein fragments may result from interrupted translation which are then released from the cell and are monitored.

Further, the present invention recognizes
20 alternately spliced forms of the mRNA for pp2c α giving rise to different sizes and/or function in different tissues and assays are designed to recognize the alternately spliced forms in the appropriate tissues.

The identification of alterations in the gene
25 product in a specific bodily fluid will indicate the source/location of a tumor. For example, with a tumor in the central nervous system, the gene product would be found in the cerebrospinal fluid. Similarly the location of other tumors or other diseases would
30 determine which bodily fluids to screen and the converse as would be known to those skilled in the art.

The present invention also provides for a kit for detecting PP2C α activity and/or alteration either at
the mRNA level or gene product level. The kit includes
35 molecular probes for mRNA for PP2C α mRNA and detection means for detecting the molecular probe and thereby the

-13-

mRNA. Alternatively, or in addition, the kit can contain probes for detecting the PP2C α gene product. The detecting means are in general are antibodies with high specificity for the gene product or agents which
5 mimic natural proteins which bind to the PP2C α gene product other agents as known in the art may also be used. The antibodies are made as described herein below and in Example 3, which specifically recognize the PP2C α or PP2C β gene products (including on the cell
10 surface) including polymorphisms thereof, and detection means for detecting the binding of the antibody thereby indicating the presence of the gene product and also distinguishing one from the other.

Where appropriate, the kits can also contain
15 antibodies directed against secondary gene products that are affected by the alteration in function of the PP2C α gene.

The present invention discloses a method of detecting cancer in a patient by detecting altered
20 levels of PP2C β gene activity compared to normal patients in a specimen isolated from a patient.

The present invention also provides for a kit for detecting PP2C β activity. The kit includes molecular probes for mRNA for PP2C α polymorphisms thereof and
25 detection means for detecting the molecular probe and thereby the mRNA or antibodies or other means of identifying alterations in the level of the gene product over normal controls as described herein.

The present invention provides an antibody, either
30 polyclonal or monoclonal, which specifically binds to a polypeptide/protein encoded by the PP2C α gene as described in Example 3 herein below. The antibodies of the present invention are used in identifying the gene product of PP2C α and PP2C β . The present invention
35 provides monoclonal and polyclonal antibodies raised against recombinantly produced PP2C α , NDDTDSASTD (SEQ

-14-

ID No:1), YKNDDTDSTSTDDMW (SEQ ID No:2), recombinantly produced pp2c β and PNKDNDGGA (SEQ ID No:3).

The present invention also provides for isolated and purified peptides NDDTDSASTD (SEQ ID No:1),
5 YKNDDTDSTSTDDMW (SEQ ID No:2) and PNKDNDGGA (SEQ ID No:3). The peptides can be produced recombinantly.

The invention further provides antibodies that will recognize the special structures at the 5'UTR or the RNA-proteins complexes responsible for the
10 controlled expression of PP2C α . Antibody which recognizes specifically the special RNA structures is also provided.

In general in preparing the antibody, either the entire pp2c α protein or peptide sequences thereof can
15 be used as an immunogen as well as polymorphisms thereof. Further, anti-idiotypic antibodies can be made against these antibodies. The antibodies may be either monoclonal or polyclonal. Conveniently, the antibodies may be prepared against a synthetic peptide
20 based on the sequence, or prepared recombinantly by cloning techniques or the natural gene product and/or portions thereof may be isolated and used as the immunogen. Such proteins or peptides can be used to produce antibodies by standard antibody production
25 technology well known to those skilled in the art as described generally in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988.

For producing polyclonal antibodies a host, such
30 as a rabbit or goat, is immunized with the protein or peptide, generally with an adjuvant and, if necessary, coupled to a carrier; antibodies to the protein are collected from the sera.

For producing monoclonal antibodies, the technique
35 involves hyperimmunization of an appropriate donor, generally a mouse, with the protein or peptide fragment

-15-

and isolation of splenic antibody producing cells. These cells are fused to a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid which has immortality and secretes the required
5 antibody. The cells are then cultured, in bulk, and the monoclonal antibodies harvested from the culture media for use.

The antibody can be bound to a solid support substrate or conjugated with a detectable moiety or be
10 both bound and conjugated as is well known in the art. (For a general discussion of conjugation of fluorescent or enzymatic moieties see Johnstone and Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, Oxford, 1982.) The binding of antibodies
15 to a solid support substrate is also well known in the art. (see for a general discussion Harlow and Lane *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Publications, New York, 1988) The detectable moieties contemplated with the present
20 invention can include, but are not limited to, fluorescent, metallic, enzymatic and radioactive markers such as biotin, gold, ferritin, alkaline phosphatase, β -galactosidase, peroxidase, urease, fluorescein, rhodamine, tritium, ^{14}C and iodination.
25 Additionally, toxins can be coupled to the antibody for targeted delivery.

The present invention also provides for transgenic human PP2C α gene and polymorphic PP2C α gene, animal and cellular (cell lines) models as well as for knockout
30 PP2C α models. These models are constructed using standard methods known in the art and as set forth in United States Patents 5,387,742, 5,360,735, 5,347,075, 5,298,422, 5,288,846, 5,221,778, 5,175,385, 5,175,384, 5,175,383, 4,736,866 as well as Burke and
35 Olson, [1991], Capecchi, [1989], Davies et al., [1992], Dickinson et al., [1993], Huxley et al., [1991],

-16-

Jakobovits et al., [1993], Lamb et al., [1993], Rothstein, [1991], Schedl et al., [1993], Strauss et al., [1993]. Further, patent applications WO 94/23049, WO 93/14200, WO 94/06908, WO 94/28123 also provide
5 information.

The present invention provides vectors comprising an expression control sequence operatively linked to the nucleic acid sequence of the PP2C α gene and portions thereof as well as polymorphic sequences
10 thereof (see Examples herein below). The present invention further provides host cells, selected from suitable eucaryotic and procaryotic cells, which are transformed with these vectors.

The vectors can be introduced into cells or
15 tissues by any one of a variety of known methods within the art. Such methods can be found generally described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1992), in Ausubel et al., *Current Protocols in*
20 *Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989), Chang et al., *Somatic Gene Therapy*, CRC Press, Ann Arbor, MI (1995), Vega et al., *Gene Targeting*, CRC Press, Ann Arbor, MI (1995) and Gilboa, et al (1986) and include, for example, stable or
25 transient transfection, lipofection, electroporation and infection with recombinant viral vectors. Introduction of nucleic acids by infection offers several advantages over the other listed methods. Higher efficiency can be obtained due to their
30 infectious nature. Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the vectors to specific cell types in vivo or within a tissue or mixed culture of
35 cells. Viral vectors can also be modified with specific receptors or ligands [Solderling, 1993] to

alter target specificity through receptor mediated events.

More specifically, such vectors are known or can be constructed by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences. Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the nucleic acids in a different form. Phagemids are a specific example of such beneficial vectors because they can be used either as plasmids or as bacteriophage vectors. Examples (see Example herein below) of other vectors include viruses such as bacteriophages, baculoviruses and retroviruses, DNA viruses, cosmids, plasmids, liposomes and other recombination vectors. The vectors can also contain elements for use in either procaryotic or eucaryotic host systems. One of ordinary skill in the art will know which host systems are compatible with a particular vector.

Recombinant methods known in the art can also be used to achieve the sense, antisense or triplex inhibition of a target nucleic acid. For example, vectors containing antisense nucleic acids can be employed to express protein or antisense message to reduce the expression of the target nucleic acid and therefore its activity. Additionally, ribozymes can be generated and used to "knock-out" the mRNA expression of the gene [Cech, 1986; Cech, 1990; Hampel et al, 1993; Sullivan, 1994].

A specific example of DNA viral vector for introducing and expressing recombinant sequences is the adenovirus derived vector Adenop53TK. This vector expresses a herpes virus thymidine kinase (TK) gene for either positive or negative selection and an expression cassette for desired recombinant sequences. This vector can be used to infect cells that have an

adenovirus receptor which includes most cancers of epithelial origin as well as others. This vector as well as others that exhibit similar desired functions can be used to treat a mixed population of cells and
5 can include, for example, an *in vitro* or *ex vivo* culture of cells, a tissue or a human subject.

Additional features can be added to the vector to ensure its safety and/or enhance its therapeutic efficacy. Such features include, for example, markers
10 that can be used to negatively select against cells infected with the recombinant virus. An example of such a negative selection marker is the TK gene described above that confers sensitivity to the antibiotic gancyclovir. Negative selection is
15 therefore a means by which infection can be controlled because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce altered forms of the viral vector or recombinant sequence,
20 cellular transformation will not occur. Features that limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are specific for the desired cell type.

25 In addition, recombinant viral vectors are useful for *in vivo* expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus
30 and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles.
35 This is in contrast to vertical-type of infection in which the infectious agent spreads only through

daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

As described above, viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. The vector to be used in the methods of the invention will depend on desired cell type to be targeted and will be known to those skilled in the art. For example, if breast cancer is to be treated then a vector specific for such epithelial cells would be used. Likewise, if diseases or pathological conditions of the hematopoietic system are to be treated, then a viral vector that is specific for blood cells and their precursors, preferably for the specific type of hematopoietic cell, would be used.

Retroviral vectors can be constructed to function either as infectious particles or to undergo only a single initial round of infection. In the former case, the genome of the virus is modified so that it maintains all the necessary genes, regulatory sequences and packaging signals to synthesize new viral proteins and RNA. Once these molecules are synthesized, the host cell packages the RNA into new viral particles which are capable of undergoing further rounds of infection. The vector's genome is also engineered to encode and express the desired recombinant gene. In the case of non-infectious viral vectors, the vector genome is usually mutated to destroy the viral packaging signal that is required to encapsulate the

-20-

RNA into viral particles. Without such a signal, any particles that are formed will not contain a genome and therefore cannot proceed through subsequent rounds of infection. The specific type of vector will depend
5 upon the intended application. The actual vectors are also known and readily available within the art or can be constructed by one skilled in the art using well-known methodology.

The recombinant vector can be administered in
10 several ways and in combination with a suitable pharmaceutical carrier. If viral vectors are used, for example, the procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site. However,
15 local administration can provide a quicker and more effective treatment, administration can also be performed by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into a spinal fluid can also be used as a mode
20 of administration, especially in the case of neurodegenerative diseases. Following injection, the viral vectors will circulate until they recognize host cells with the appropriate target specificity for infection.

An alternate mode of administration of a PP2C α vector can be by direct inoculation locally at the site
25 of the disease or pathological condition or by inoculation into the vascular system supplying the tumor with nutrients. Local administration is advantageous because there is no dilution effect and,
30 therefore, a smaller dose is required to achieve expression in a majority of the targeted cells. Additionally, local inoculation can alleviate the targeting requirement required with other forms of administration since a vector can be used that infects
35 all cells in the inoculated area. If expression is desired in only a specific subset of cells within the

inoculated area, then promoter and regulatory elements that are specific for the desired subset can be used to accomplish this goal. Such non-targeting vectors can be, for example, viral vectors, viral genome, plasmids, phagemids and the like. Transfection vehicles such as liposomes can also be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

10 In a preferred embodiment, a virus vector based on modified AAV is used. AAV has been shown to integrate into the human genome in chromosome 19q13.3. Alteration of the AAV genome in a mode that will allow it to integrate in a site specific manner into the PP2C α regulatory region is used. (see Example 7)

15 The invention further provides a method of treating cancer including the steps of first determining the type of cancer and cells expressing the cancer and then preparing a vector as described herein above which will specifically target the cancer cells and includes regulatory elements to control the expressibility of PP2C α . The vector is then administered to the patient and can include a suitable pharmaceutical carrier which will not affect bioactivity of the vector. Alternatively an antisense vector can be prepared and used to control the expression of PP2C α .

pp2c is a protein serine/threonine phosphatase [Cohen 1989]. It is unique among phosphatases since it requires magnesium and is not sensitive to certain phosphatase inhibitors such as okadaic acid [Cohen 1991]. The pp2c family consists of two cytoplasmic isoenzymes in mammalian tissues [McGowan and Cohen, 1987] and at least three pp2c-like enzymes in yeast show the same enzymatic and biochemical properties. The two mammalian isoenzymes are monomers but differ

slightly in molecular mass (44KDa and 42KDa) and are designated pp2c α and pp2c β . There is 70% homology between the α and β isoforms. At the carboxy terminal of the pp2c α there is a fifteen amino acid sequence
5 which differs from pp2c β . In humans the sequence is YKNDDTDSTSTDDMW (SEQ ID No:2).

A 106kb cosmid coding for pp2c α and additional proteins FosB and ERCCI has been sequenced [Martin-Gallardo et al., 1992] (GENBANK accession
10 number: M89651). Further, the cDNA sequences of PP2C α in humans is known [Mann et al., 1992]. However, attempts to align the 5'UTR of the cDNA with the genomic sequences were not successful.

A hypothesis for the above observations can be
15 made, but it is not to be construed as limiting the present invention to this one mode. Applicants propose that the UTR consists of several small exons with large introns and propose that PP2C α and FosB have a common regulatory region (ERCCI may also share the regulatory
20 region). Alternatively, it is possible that PP2C α is a very large gene and that the 5' end and the control region do not reside within the 106kb cosmid in a region located 5' to the 106kb cosmid. As a further alternative, the region of 9kb from the cosmid was not
25 sequenced due to the high G/C content and it may contain the 5'UTR region and the promoter.

In a preferred embodiment the AAV virus and/or CHINT or other regulatory sequences related to the PP2C α gene are used in the vector, particularly those
30 which are used to treat patients. CHINT is a cellular sequence which was recombined into the AAV in 9-3 cells; the sequence is set forth in Table 5 (int.li; SEQ ID No:19). The vector can either integrate into the regulatory control of PP2C α and alter its
35 expression in the same way as AAV alters cells into which it integrates as it is an oncosuppressive virus.

This thereby reinstates normal cellular growth, or there is growth arrest or there can be activation of programmed cell death - apoptosis pathway depending on the cell type, type of cancer, stage of
5 differentiation, and other factors as known to those skilled in the art. [Schlehofer, 1994]

There are several elements in AAV and/or CHINT or in the PP2C α regulatory elements that can be used to control PP2C α expression taking advantage of the
10 following observations:

1. PP2C α has a very long 5' and 3' UTR (they are larger than the coding capacity). Specific folding of the RNA and interaction with specific sets of proteins might effect its expression dramatically. At certain stages
15 there might different modes of folding and these different proteins may interact with the RNA and alter its expression.
2. The CHINT sequences involved in the integration have very interesting motifs which might be used for the
20 site specific integration. Moreover, applicant has data demonstrating that specific AAV sequences adjacent to the AAV integration site are responsible for the suppression of DNA amplification. These sequences can be used in vectors as a therapeutic and are described
25 herein below as silencer (SEQ ID No:13) and mini-silencer (SEQ ID No:14).

The invention further provides a method of treating cancer by using an AAV based vector or other vector for cancer treatment that only functions
30 specifically in cells in which PP2C α is improperly activated. The vector is administered to those who have been diagnosed with a tumor as is known to those skilled in the art. The AAV vector (or other regulatory factor as disclosed herein) in one
35 embodiment is under the control of a promotor, rep, that is expressed in transformed cells. The integrated

-24-

vector will control PP2C α expression in the cell reversing transformation as shown in the Examples. Further, the vector will be targeted to the cell type that has been transformed.

5 Further, since the gene product of PP2C α is expressed on the cell surface, antibody directed against the gene product can activate/inactivate the expression of the gene via the signal transduction pathway. Therefore antibodies can be used
10 therapeutically to treat patients in which the PP2C α gene needs to be re-regulated. The use of Fab fragments and other means known in the art can be used to insure that the antibodies upon administration to a patient do not have secondary unwanted effects.
15 Alternatively, a ligand or other molecule which can specifically bind to the PP2C α gene product can be used. The present invention therefore provides a method of binding the gene product of PP2C α expressed on the surface of a cell to induce signal transduction
20 thereby suppressing the transformed phenotype.

The invention further provides a method of treating diseases due to aberrant phosphorylation due to alteration of expression of PP2C α by controlling PP2C α expression. Such disease can be neurologic. For
25 example, behavioral changes could be associated with aberrant phosphorylation. As shown by Brown et al, 1996 mutations in fosB can lead to behavioral changes. As discussed herein above, fosB and PP2C α are on the 106kD cosmid. There is some indication that they may
30 be co-regulated. Therefore aberrant expression of PP2C α can be expressed as behavioral changes. Further, the levels of PP2C α activity are extremely high in cardiac and kidney tissues compared to other tissues. Therefore alterations in PP2C α activity will be
35 reflected in these tissues.

-25-

The present invention provides a method of suppressing gene amplification by interrupting the binding or action of DNA polymerase α primase and RNA polymerase II with the gene product of PP2C α by
5 preparing an antisense vector which will specifically target the binding region of DNA polymerase α primase and RNA polymerase II to the PP2C α gene product and delivering the vector to the cells as based on the observations set forth in Example 9. Applicants have
10 observed that in tumor cells pp2c α binds to the CTD domain of RNA polymerase II. Therefore alternatively, delivery of a peptide with the CTD domain can be used via competitive binding strategies to control the binding leading to gene amplification.

15 To investigate the role of AAV in tumor suppression an AAV/neo virus JDT277 was introduced into SV40 transformed Chinese hamster (CO60 and OD4 cells) and mouse mammary tumor cells (DA3). CO60 is a cell line of SV40 transformed Chinese hamster embryo cell
20 lines [Lavi, 1981]. The OD cell line was established by transfection of Chinese hamster embryonic cells with origin deleted SV40 DNA [Lavi, 1985]. The mouse DA3 cell line was derived from mammary tumors syngeneic to BALB/c mice [Sotomayor et al, 1991]. JDT277 virus
25 contains the portion of the AAV2 genome, which encodes the viral Rep proteins, the AAV terminal inverted repeats (TIRs) and the prokaryotic neomycin phosphotransferase gene (neo), conferring resistance to G418. The neo gene was inserted at nucleotide 1882,
30 resulting in carboxy terminus truncated Rep proteins. The truncation of the rep proteins does not affect the ability of the AAV/neo virus to replicate in Adenovirus coinfecting human cells.

Single colonies were isolated and amplified by
35 serial passages in the presence of G418. The resistant cells were designated 9-1 to 9-5 for cells derived from

-26-

CO60 cells and A20-A29 for cells derived from OD4 cells. The cell lines derived from the mouse cells DA3 were designated J1-J15.

Alterations of the transformed phenotype:

5 Following AAV integration the cells lost several of their transformed characteristics.

1. Suppression of SV40 DNA amplification. (Example 7)

A characteristic trait of tumor cells is their capability to amplify DNA. CO60 cells are used as a
10 model system to study gene amplification and SV40 amplification can be induced in the cells as a results of treatment with carcinogens [Lavi, 1981; Aladjem and Lavi, 1992]. Following AAV/neo virus integration, the cells were incapable to amplify SV40. Most AAV/neo
15 cell lines derived from CO60 cells lost their capability to amplify SV40 upon treatment with carcinogen in contrast to the parental CO60 cells [Tal Burstyn, 1993]. Extracts from AAV/neo cells derived from both OD4 and CO60 cells lost their capability to
20 amplify SV40 in vitro [Winocour et al., 1992; Tal Burstyn, 1993].

2. The cells harboring the integrated SV40 became highly sensitive to treatment with UV or MNNG. [Winocour et al., 1992].

25 3. The transformed cells lost their capability to grow in soft agar, a characteristic typical to transformed cells.

4. The cells displayed apoptotic phenotype as measured by

30 a) the cell cycle pattern was altered and apoptotic cells appeared spontaneously in the AAV/neo cells and the level was enhanced following treatment with DNA damaging agents. (Figure 1A, Table 3A and Figure 1B, Table 3B),

35 b) the condensation and fragmentation of the chromatin and cytoplasm. Condensation and

fragmentation of chromatin was monitored by staining with acridine orange. Ethidium bromide did not stain these cells.

Acridine orange is taken up by both live and dead cells creating fluorescent-green signals, whereas ethidium bromide is only taken up by nonviable cells and gives bright red fluorescence. This double staining system provides a means to distinguish between dead and living cells, and cells that underwent apoptosis before they lost their membrane integrity. Both normal or apoptotic nuclei in living cells fluoresce bright green. In striking contrast, normal and apoptotic nuclei in dead cells fluoresce bright red.

A substantiated fraction of the cells displayed apoptotic nuclei showing condensed chromatin upon staining with acridine orange. In addition, the cells displayed a strong shrinkage of the cytoplasm. Often the nuclei were disrupted into a multitude of micronuclei. These cells underwent apoptosis without losing their membrane integrity. EtBr did not penetrate into these cells, thus the cells were still alive. A large amount of living apoptotic nuclei were found in the treated AAV positive cells compared to a considerably lower percentage in the treated (7.5 µg/ml MNNG) and control CO60 cells. The same pattern of staining repeated in all the AAV/neo cell lines. Hence, this apoptotic phenotype was a common feature to all the AAV/neo cells.

c) by the breaks in the chromosomal DNA:

Programmed cell death was shown to be associated with DNA fragmentation. This approach to detect apoptosis was based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to 3'-OH ends of DNA, ensuing a synthesis of polydeoxynucleotide polymer [Gavrieli et al., 1992]. By adding

-28-

biotinylated deoxyuridine to fixed cells, TDT was used to incorporate these nucleotides at sites of DNA breaks. The signal was amplified by FITC-Avidin binding enabling identification by fluorescence microscopy. In all AAV/neo cell lines, Applicants could detect a distinct pattern of nuclear staining, directly correlated to the typical degradation of chromatin in apoptotic cells. Since this reaction is specific, only the apoptotic nuclei are stained. As a positive control for the efficiency of the technique, Applicants used CO60 nuclei treated with DNase.

C9-2 and C9-3, the AAV positive clones displayed a bright nuclear fluorescence 48 hours after treatment with 2.5 μ g/ml MNNG, whereas the controls without any treatment showed only a low background fluorescence. Applicants could see the characteristic degradation of the nuclei into a multitude of small highly fluorescent micronuclei. The fluorescence obtained was similar to the positive control.

Untreated AAV/neo cells and control and treated CO60 (7.5 μ g/ml MNNG) did not show any sign of fluorescence. This pattern of nuclear degradation appeared in all AAV/neo cell lines tested (approximately 20), however, the extent of fragmentation varied in the different lines.

Unexpectedly, the revertant cells, designated C9-3-2 and C9-3-12, which were selected on the basis of loss of resistance to G418, and lost their integrated AAV sequences [Burstyn, 1993], still maintained their apoptotic phenotype following treatment with 2.5 μ g/ml or 5 μ g/ml MNNG.

Further analysis by FACS, Giemsa staining, and electrophoretic separation of the nucleosomal DNA fragments from high molecular weight cellular DNA supported these results.

The integrated AAV in the AAV/neo cells (Examples 6 and 7)

Analysis of cell extract derived from the Chinese hamster A20-A29, 9-1 to 9-5 and the mouse DA3 derivatives for the expression of rep proteins by immunoblotting with anti-Rep antibodies demonstrated that in all cell lines the authentic Rep products were not present. In some cell lines a short protein, probably a truncated protein, reacted with the anti-Rep antibodies [Winocour et. al., 1992].

PCR analysis of most cell lines for the presence of the intact rep promoter region demonstrated that in most cell lines the promoter region of the Rep protein was reorganized as a result of deletions, insertions and rearrangements of the AAV sequences thus eliminating the expression of the authentic Rep proteins.

Applicant focused on the analysis of one Chinese hamster cell lines, 9-3, derived from CO60 cells (Figure 3). The integrated AAV undergoes duplication in this cell line and the chromosome harboring the AAV contains two regions in which AAV is integrated. This duplication of AAV probably resulted from the massive rearrangement which occurred in the Chinese hamster genome following AAV integration. In all Chinese hamster cell lines studied so far by *in situ* hybridization (6 independent cell lines) the chromosome harboring the integrated AAV was altered and was different in many respects from all the typical Chinese hamster chromosomes, thus the identity of the chromosome could not be established.

The integrated AAV and flanking cellular sequences for 9-3 were cloned into a phage (Figure 3). As diagramed in Figure 3 the viral genome underwent several changes. Sequences downstream to the AAV p5 promoter were deleted and replaced by a cellular

fragment "CHINT". In addition, deletions and rearrangements in the 5' portion of the AAV/neo genome were observed. In contrast, the region coding for the Neo gene and the 3' end of the viral genome remained
5 intact. (Similar alterations were observed in all AAV/neo Chinese hamster and mouse cell lines tested).

The "CHINT" sequences were used as a probe to analyze AAV integration site in different AAV/neo Chinese hamster clones (Figure 2). In several AAV/neo
10 clones there was a shift in this fragment suggesting that the size of this fragment was altered upon AAV integration. Some of the shifted bands also hybridized to AAV probe demonstrating that AAV indeed integrated into this region. A probe from the subcloned plasmid
15 pSL9-6 derived from the flanking cellular sequences (Figure 3) was hybridized to the mouse DA3 AAV/neo cells and in most cases it was associated with AAV integration. These results suggest that the AAV integration site in Chinese hamster might be similar to
20 the one in mouse cells.

Sequence comparison using Genetic Computer Group Inc. software demonstrated that the CHINT sequences are 58.3% homologous to a sequence in the human chromosome 19q13.3. This human sequence is a part of a 106Kb
25 fragment which was automatically sequenced and analyzed [Martin-Gallardo et al., 1992] (GENBANK accession number: M89651). The region which showed homology to the CHINT sequences was a part of the gene coding for human type protein phosphatase 2C (pp2c α). According
30 to the cDNA sequence of this gene the exact region homologous to CHINT is located upstream to the 5'UTR of PP2C α (Table 5) (GENBANK accession numbers: human PP2C α S87759, rabbit PP2C α S87757).

Using a PCR fragment derived by using two primers
35 (Primers 1 and 4, Methods herein below) for PP2C α , cDNA DNA from 9-3 was probed and a XbaI fragment was found

-31-

which hybridized to AAV, CHINT and PP2C α , and an *EcoRI* fragment was found which hybridized to PP2C α and CHINT in CO60 DNA. Thus, PP2C α is indeed localized in very close proximity to CHINT in CO60 cells and is at the integration site in 9-3 cells. In situ hybridization confirmed this conclusion (Figure 4A).

In both Chinese hamster (CO60 and OD4) and mouse cells (DA3) a portion of the PP2C α sequences was adjacent to the CHINT hamster sequence present in pSL9-6 (Figure 3) which was derived from the lambda clone of the AAV integration site. Thus in both the normal Chinese hamster and mouse chromosome PP2C α is indeed localized at very close proximity, less than 4Kb from the sequences surrounding the integrated AAV (Figure 4B).

Furthermore, in mouse DA3 cells harboring the integrated AAV genome the AAV sequences were associated with PP2C α or with fragment 6 or with both (Figure 5). The integrated AAV was cloned from DA3J7 (λ DA37A) and parts of the phage were sequenced as shown in the figure. Homology was found to a region within the integrated AAV in the λ SL9-1 plasmid (Figure 3) and to the human chromosome 19q13.3 in the position 23467 - 23715 in cosmid MMDA (Access #M63796) which was automatically sequenced and contained PP2C α first coding exon in position 59770 in MMDBC, GenBank accession #M89657, as well as 35-3.seg and 35-T7 as shown in Figure 3. MMDA and MMDBC are two cosmids in the same contig. (More details on the sequences are found in Example 10 herein below).

Little is known about the role of PP2C α in the cell or about its native substrates mainly because of the lack of specific inhibitors for PP2C α . In *Schizosaccharomyces pombe* it has been shown that pp2c α -like enzymes are important for heat shock response and in osmoregulation [Shiozaki, 1995;

-32-

Shiozaki, 1994]. In *Saccharomyces cerevisiae* pp2c-like activity has been implicated in the regulation of tRNA splicing and cell separation [Robinson et al., 1994]. In neural cells pp2c α might have a role in the regulation of the Ca⁺⁺-independent activity of Ca⁺⁺/calmodulin dependent protein kinase II [Fukugana, 1993]. Otherwise, information about pp2c α is scarce.

pp2c α might be a cell marker itself. The prior art does not provide information about pp2c α expression in tumor cells. There is a publication suggesting that pp2c α might have a role during myogenic differentiation. [Ohishi, 1992]. Based on the presence of a 10 amino acid motif which appears also in other transcription factors, pp2c α might function like a transcription factor and might regulate transcription in the cell under specific growth conditions and tissues. It can thus behave like E2F which is a major transcription factor and can act when improperly expressed either as an oncogene or as a tumor suppressor factor [Weinberg, 1996]

Unexpectedly, analysis of the PP2C α mRNA in the AAV/neo cells demonstrated that the transcription of the gene was reduced upon treatment with DNA damaging agents in contrast to the parental SV40 transformed cells in which PP2C α was induced following the treatment (Figure 6).

The following is the densitometry analysis of the hybridization signals

	CO60 C	CO60 T	$\frac{\text{CO60 T}}{\text{CO60 C}}$	C9-3C	C9-3T	$\frac{\text{C9-3T}}{\text{C9-3C}}$
PP2C α rRNA	0.15	0.31	2.06	0.26	0.15	0.57

The transcription of PP2C α in C9-3 was reduced upon treatment with MNNG in contrast

-33-

to the parental SV40 transformed cells in which PP2C α was induced following treatment.

The intact cDNA coding for pp2c α was cloned from a
5 cDNA library (provided by M. Oren, the Weizmann
Institute of Science, Rehovot) and PP2C α clone was
stably introduced into the AAV/neo cells which lost the
transformed phenotype following AAV-integration. The
transformed features were rescued following the
10 expression of the PP2C α clone in the PP2C α neo cells,
the cells regained the properties of transformed cells,
grew in soft agar and lost their apoptotic phenotype,
but the cells could not be propagated. Similar cells
which were transfected in the same efficiency with a
15 control plasmid containing a truncated cDNA of non
relevant gene did not regain the capability to grow in
soft agar.

From these studies it is apparent that PP2C α has a
key role in the initiation and/or maintenance of
20 transformed cells. It should be noted that though the
cells grew in soft agar applicant was not able to
isolate viable cells suggesting that unbalanced
expression of the cells led to aberrant growth and to
cell death.

25 PP2C α appears to be important in development. The
high conservation of the gene and protein throughout
evolution, the specific control signals including IRES
(internal ribosome entry site) at the 5'UTR support
this. The findings by other laboratories that AAV
30 infection effects specifically tumor cells might have
two explanations: 1) The virus does not infect normal
cells or cannot integrate into their genome in a
specific manner. 2) Alternatively, if AAV integrates
into PP2C α in normal cells the disruption of this gene
35 might not effect them or might be lethal not allowing
the survival of such cells. The fact that the

-34-

inactivation of only one allele of PP2C α is responsible for the changes in the transformed phenotype and that the introduction of a functional PP2C α cDNA clone rescues the transformed phenotype demonstrates the importance of PP2C α . Applicant also noticed that in highly tumorigenic Chinese hamster cells derived from 9-3 that the whole chromosome carrying PP2C α is duplicated 3, 4, and even 5 times.

In human, on the same chromosome, in close proximity to PP2C α there is an important tumor specific marker called cancer embryonic antigen (CEA), which appears in most tumor cells. Both genes are mapped to chromosome 19q13.3. Targeting the treatment to cells carrying a marker like CEA should help. (It might be possible that CEA by itself is not relevant to cancer but it is associated with the enhanced expression of PP2C α or duplication of this chromosomal region contains both genes).

The above discussion provides a factual basis for the use of PPC2 in cancer detection and therapy. The methods used with and the utility of the present invention can be shown by the following non-limiting examples and accompanying figures.

EXAMPLES

METHODS

General Methods in Molecular Biology: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1992), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1994). Polymerase chain reaction (PCR) was carried out generally as in *PCR Protocols: A Guide To*

Methods And Applications, Academic Press, San Diego, CA (1990).

Reactions and manipulations involving other nucleic acid techniques, unless stated otherwise, were performed as generally described in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, and methodology as set forth in United States patents 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057 and incorporated herein by reference.

Antibody Capture Assay: The steps of the method are: (1) binding of antigen to a solid phase; (2) binding of the antibody to the antigen; and (3) binding of a labeled secondary antibody to the complex. By binding constant amounts of rpp2c α to the solid phase, Applicants have used this technique to detect and quantitate monoclonal antibodies during the rounds of cloning, and to compare polyclonal antibodies from different rabbits and bleedings. The assay has also been used to detect pp2c α in crude extracts of tissues and cell cultures.

Immunoblotting: The steps of the method are: (1) preparation of antigen sample; tissue extracts, cell culture extracts or rpp2c α preparations; (2) resolution of the sample by SDS-PAGE; (3) transfer of the separated proteins to a nitrocellulose membrane; (4) blocking nonspecific sites on the membrane; (5) incubation with poly- or monoclonal antibody; and (6) detection by labeled secondary antibody. Applicants have used immunoblotting for characterization of antibodies described herein above and for detection of pp2c α in cell and tissue extracts. [Harlow and Lane]

Immunoprecipitation: The method steps are: (1) immobilization of monoclonal antibodies to a solid matrix (anti-mouse IgG conjugated agarose); (2) binding of antigen to immobilized antibodies; (3) resolution of

bound proteins on SDS-PAGE; and (4) immunoblotting and detection of antigen by affinity purified rabbit polyclonal antibodies. The method has been used to estimate the amount and the molecular mass of different sized pp2c α and β polypeptides that were discovered.

5 pp2c α Activity Assay: PP2C α gene product is purified from the mouse cells by general procedure, and its activity is assayed by its ability to dephosphorylate [32P] casein [McGowan and Cohen, 1988].

10 Oligonucleotide Primers for Reverse PCR: Rat PP2C- α cDNA specific primers were used for reverse transcription and PCR. These primers were obtained from General Biotechnology, Rehovot, and used without further purification. The primers' position is

15 according to the rat kidney nucleotide sequence of PP2C α cDNA reported by Tamura et al., [1989] in the Genbank (accession number: Gb_ro: Ratpp2c, J04503). The approximate position of each primer on the rat PP2C α cDNA:

20 Primer #1: 5' - AGGATCAAGTCATAATGGGA - 3' (74-93nt - sense) (SEQ ID No:4)
Primer #4: 5' - GCTGGAGTCTGATTTACAAC - 3' (1454-1473nt -anti sense) (SEQ ID No:5)

Antisense RNA: Artificial antisense RNA complementary to the PP2C α gene is synthesized, and transfected to the mouse cells by the method of Inouye [1988].

25 Identification Of Unique Changes In Gene Expression By Differential Display: In order to detect changes in the expression of cellular genes mediated by AAV

30 integration, the differential display method is used [Liang and Pardee, 1992; McClelland et al., 1995]. This method is directed toward the identification of differentially expressed genes among approximately

35 15,000 individual mRNA species in a pair of mammalian cell population such as infected and uninfected cells, and recovering their cDNA and genomic clones. The

-37-

strategy of the method consists of the following steps:

(1) Reverse transcription in fractions using a set of anchored primers, (2) amplification of cDNA species from each fraction using a set of arbitrary primers and anchored primers by labeled PCR, (3) electrophoretic separation of the resulting fragments on sequencing gel, (4) reamplification of fragments that are different in the two situations, cloning and sequencing, and (5) confirmation of differential expression by an independent RNA analysis technique.

More specifically, total RNA is isolated from cells as described by Sambrook et al. The RNA is reverse transcribed with an oligo dT primer designed to bind to the 5' boundary of the poly A tail. The cDNA is amplified in a PCR reaction with the oligo dT primer and a second 10-mer arbitrary in sequence. 40 cycles of PCR are done in the presence of [35S]-dATP, in the following conditions: 94°C for 30 seconds, 42°C for 60 seconds and 72°C for 30 seconds. The amplified cDNAs are separated on a 6% sequencing gel, then exposed to X-ray film.

Bands of interest (bands that are differentially displayed) are cut out from the gel, and reamplified with the same primers as used to generate the original PCR product. To confirm differential regulation of individual candidate bands, Northern blot hybridizations is performed. Fragments of interest are cloned using a TA cloning Kit, and sequenced. Genes detected by this method are hybridized to Northern blots from the appropriate cells.

Chromatin Structure In Latently Infected Cells: Higher order chromatin structure may affect the transcription of cellular genes. Judging by their increased sensitivity to digestion with DNaseI or micrococcal nuclease, transcriptionally active chromatin regions are less tightly packed than chromatin containing

-38-

transcriptionally inactive genes. Chromatin is partially purified and digested by micrococcal nuclease [Roth et al., 1990]. Purified DNA fragments are digested with a unique restriction enzyme to generate a series of fragments with one end defined by micrococcal nuclease and the other defined by the restriction enzyme. Fragments are separated by agarose gel electrophoresis, transferred to nitrocellulose filters and probed with labeled DNA fragments. Naked DNA is purified and processed similarly. Nucleosome position and nuclease sensitive regions are inferred by comparison of fragments from naked DNA and chromatin.

The methylation state of genes can indicate chromatin changes. Gene specific DNA methylation is measured by the methylation assay [Kafri et al., 1992]. In this method, total cellular DNA is digested with methyl-sensitive enzymes, such as *HpaII* or *HhaI*, and specific fragments of DNA that contain these sites are amplified by flanking oligonucleotide primers. If a specific site is methylated, the amplification will proceed normally. On the other hand, the presence of an unmethylated site will result in digestion of the fragment and the subsequent failure to visualize the amplification product. When properly calibrated, this assay is linear over a wide range of DNA concentrations and can be used to accurately measure the degree of DNA methylation at specific sites.

EXAMPLE 1

EXAMPLE OF STUDIES WITH AAV

A. Infection of mouse cells with AAV:

A1. Generation of mouse cells with stably integrated AAV:

DA3 cells were infected with the JDT277 AAV/neo hybrid virus according to Winocour et. al. [1992], with slight modifications. Single colonies were isolated

and amplified by serial passages in the presence of the antibiotic G418. The resistant cell lines were designated DA3J.

5 The DA3 cell line was derived from the *in vivo* D1-DMBA-3 mammary tumor syngeneic to BALB/c mice. The DA3 cell line produces tumors in BALB/c mice with the same growth kinetics and expresses the same tumor associated antigen (Ag) on its surface as the parental tumor. The cells express specific markers for tumor
10 cells, and cease to express specific Ag typical to normal breast cells [Sotomayor et al., 1991].

To assess the influence of AAV on mouse cells, DA3 cells were infected with the JDT277 AAV/neo hybrid virus [Tratschin, 1985]. JDT277 contains the portion
15 of the AAV2 genome, which encodes the Rep proteins, the AAV terminal inverted repeats (TIRs) and the prokaryotic neomycin phosphotransferase gene (neo), conferring resistance to G418. The neo gene was inserted at nucleotide 1882, resulting in carboxy
20 terminus truncated Rep proteins. The truncation of the Rep proteins does not affect the ability of the AAV/neo virus to replicate in Adeonvirus coinfecting human cells.

Single colonies were isolated and amplified by
25 serial passages in the presence of G418. The resistant cells were designated DA35.

A2. Characterization of the AAV genome in the DA3J cells:

a. Southern analysis

30 Genomic DNA isolated from DA3J1 - DA3J7 clones was digested with different restriction enzymes (BglII or EcoRI), electrophoresed and hybridized to radiolabelled AAV DNA. The hybridization pattern is different in each clone, probably due to rearrangement of the AAV
35 genome. Indeed it is known that integration of AAV DNA

-40-

is frequently accompanied by alterations within the viral sequences [Walz and Schlehofer, 1992].

b. PCR analysis

To find whether the rep promoters and ORF's were present in the DA3J cell lines, PCR reactions were carried on 13 clones (DA3J1 - DA3J13), using different oligonucleotide primers complementary to the AAV and neo sequences. The results demonstrated that parts of the viral sequences in each clone were somehow interrupted. In all the examined cell lines the AAV rep ORF's were not intact, thus, impairing the expression of the AAV specific proteins.

In two clones, DA3J3 and DA3J4, two AAV molecules integrated into the host genome in a head-to-tail pattern. This finding is in agreement with earlier studies showing that the AAV DNA recombined into the Chinese hamster host DNA, at least in some cases, as a head-to-tail concatamer of more than one viral genome, via the terminal sequences of the viral molecule [Cheung et al., 1980; Walz and Schlehofer, 1992]. The integrated AAV from DA3J7 served as a silencer for SV40 replication in 293 cells in a similar assay to that described in Example 7 herein below.

A3. Expression of AAV genes in the infected DA3J cells:

Cell extracts from the infected DA3 were prepared according to Winocour et al. [1992]. Samples from the extracts were electrophoresed on a 12% PAGE, and immunoblotted with an anti-Rep antibody. The results showed that, the two major Rep proteins, Rep78 and Rep68, are not expressed in any of the infected cells, however one of the small Rep proteins, Rep40, is expressed in two clones, DA3J11 and DA3J13. These results were expected from the PCR analysis that showed that in all the examined cell lines the rep ORF's were not intact.

B. Site specific integration

B1. Comparison of the integration site of AAV in the mouse cells with the AAV integration site in the Chinese hamster cells:

5 Genomic DNA from parental DA3 and DA3J clones (DA3J1 - DA3J7) was digested with *Bgl*III or *Eco*RI. Following electrophoresis the blots were hybridized once with the cellular sequence from the virus/cell junction, isolated from C9-3 (psL9-6), and once with
10 radiolabelled AAV DNA. In three of the cell lines (DA3J3, DA3J4 and DA3J6) the cellular probe and the AAV probe hybridized to common bands. Using PP2C α probe applicant found in *Bgl*III digested DNA that both AAV and PP2C α hybridized to the same bands. Thus, both in
15 Chinese hamster and mouse, AAV always integrated into the same site in the vicinity of PP2C α . Note that in all cell lines including the parental DA3 cells, PP2C α and the cellular clone 6 probes hybridized to the same band which is the preintegration site.

20 C. Effect of AAV on the cellular phenotype:

 The following cytological properties were compared between DA3 infected and parental cells:

a. Plating efficiency

25 As shown in Table 1, the plating efficiency of the DA3J cells was reduced compared to the plating efficiency of the parental DA3 cells, by 11% (DA3J2) to 54% (DA3J3).

b. Sensitivity to UV irradiation

30 As shown in Table 2, the DA3J cells show increased sensitivity to UV irradiation compared to the parental DA3 cells. There is a decrease of 5% to 55% in the survival rate of the DA3J cells compared to the DA3 cells.

35 These results are in agreement with other studies, which demonstrated that AAV infected cells (Hela, CO60) show reduced plating efficiency, and enhanced

-42-

sensitivity to UV irradiation, compared to uninfected cells [Walz and Schlehofer, 1992; Winocour et al., 1992].

5 It is interesting to note that, DA3J3 shows the lowest plating efficiency, and the highest sensitivity to UV irradiation. This may be due to the fact that DA3J3 contains two integrated AAV molecules, while DA3J1 and DA3J2, contains only one.

c. FACS analysis

10 FACS analysis was performed on DA3, DA3J1, DA3J2, and DA3J3 as described by Vindelov et. al., [Vindelov et al., 1983]. In this procedure no significant changes were observed between the parental DA3 cells and the DA3J cells.

15

EXAMPLE 2

CLONING AND EXPRESSION OF PP2C α

Full length of the coding region of PP2C α cDNA was isolated from a rat cDNA library. The cDNA was cloned
20 into the expression vector pET-17b (pET System, Novagen) between the KpnI and NotI restriction sites. *E. coli* cells (BL21-DE3) transformed by the expression plasmid yielded high levels of recombinant pp2c α (rpp2c α) as observed by a very prominent ~45kDa band on
25 SDS-PAGE.

Assay of pp2c α activity: Protein phosphatase activity was found in crude extracts of cultures harboring the recombinant plasmid, as measured by the method of McGowan and Cohen [Methods Enzymol. 159: 416-
30 429, 1988].

Purification of rpp2c α : Overnight cultures were grown at 30°C in LB medium containing ampicillin. The cells were harvested and disrupted by sonication. The sonicate, cleared by centrifugation, was further
35 purified by ammonium-sulfate precipitation and anion exchange chromatography on DEAE-sephadex.

EXAMPLE 3**PRODUCTION AND ANALYSIS OF ANTIBODY**

Polyclonal Antibody preparation in rabbits: Crude cell extracts containing ~250-500 μ g rpp2c α were separated on 12% preparative SDS-PAGE (200x150x1.5 mm). The rpp2c α band, located by side-strip staining, was excised and stored at -20°C. For injection in rabbits, the band was fragmented by repeated passage through a 18 gauge syringe needle and mixed with an equal volume of Freund's adjuvant. Four ml of emulsion originating from one SDS-PAGE band were used to inject two rabbits. The rabbits, which were pre-bled, were injected subcutaneously at four week intervals. For primary immunization complete Freund's adjuvant was used and all other injections were in incomplete Freund's. The animals were bled every two weeks and the serum was cleared by centrifugation. The antibodies were designated 351 and 343. Unless otherwise designated work described herein used 351.

Monoclonal antibody preparation: Purified rpp2c α was used for monoclonal antibody preparation, by mouse hybridoma production as described herein above. Hybridoma colonies were screened by antibody capture assay (see herein below) and by immunofluorescent cell staining. Positive colonies were subjected to two rounds of cloning and screening by the same methods. Finally, eight (8) positive clones were chosen for further study. Antibodies from these clones were collected as tissue culture supernatants and also as ascitic fluid.

Antibodies Developed Against pp2c α and pp2c β

(1) A rabbit polyclonal designated 801 raised against the carboxy terminal peptide (10 a.a.). This antibody recognizes pp2c α and not pp2c β .

-44-

The antibody was raised in rabbits and was affinity purified against the rpp2c α . This antibody was used in most of the histochemical analyses.

5 A rabbit polyclonal antibody raised against PNKDNDGGA (SEQ ID No:3), the carboxy terminal of pp2c β .

(2) A rabbit polyclonal designated 351 raised against rpp2c α which recognizes epitopes on both α and β .

10 (3) Eight independent monoclonal antibodies which were raised against the rpp2c α and were screened and chosen according to their reaction in ELISA with the rpp2c α and by their capability to recognize pp2c (α and β or α) by immunofluorescence staining of hepatoma cells by Western blotting and immunoprecipitation.

15 Table 4 provides the characterization of eight monoclonal antibodies by antibody capture assay, immunoblotting and immunoprecipitation. The combination of these assays allow the isolation of monoclonal antibodies with the proper specificity of
20 the present invention.

EXAMPLE 4

Expression of pp2c α in normal Breast tissue and Breast tumors: Paraffin blocks obtained from normal breast
25 and breast carcinoma were stained with 801 antibodies specific to pp2c α and then with secondary antibodies coupled to peroxidase. The substrate was DAB. The samples were counterstained with methylene blue. In a few experiments, the antibodies used were monoclonal
30 2A3 which are specific to pp2c α . The magnification was $\times 400$. Normal liver and hepatoma tissue and normal colon and colon carcinoma tissue was also tested.

In the normal and hyperplastic breast samples, the nuclei were stained very predominantly with the
35 antibodies. In breast carcinoma, there was a predominant staining in the cytoplasm. In invasive

carcinoma, no staining with anti-pp2c α was observed. Interestingly, in liver tissue cultured cells, when stained with 801 or 2A3 antibodies, the cell surface was stained indicating that PP2C α gene product was
5 expressing in the cell membrane. In normal liver cells a very pronounced cytoplasmic staining with few very strong nuclear regions stained. In the hepatoma fainter cytoplasmic staining and less staining in the nucleus was observed.

10 It should be noted that it appears there is a differential loss of pp2c α as the cells are advanced in their malignant appearance in the counterstaining.

EXAMPLE 5

15 Expression of pp2c α in normal Colorectal tissue and Colorectal tumors: The level of the protein phosphatase 2C α (pp2c α) expression was assessed in colorectal cancer tissues in comparison to normal colon tissues and in Chinese hamster embryo (CHE) cell line
20 in comparison to the non permissive SV40 transformed Chinese hamster cells (CO60), by the reverse transcriptase polymerase chain reaction assay (RT-PCR).

Samples of 1 μ g total RNA were denatured at 65°C for 10 minutes in the presence of 0.5M oligo dT (15
25 mer) as an anti-sense primer, and immediately chilled on ice. First strand cDNAs were obtained after 60 minutes at 37°C in a 50 μ l reaction mixture containing: 0.25mM dNTPs (Promega), 10mM DTT, 20u RNasin, 50u MMLV reverse transcriptase and 5 μ l of 10 x reaction buffer
30 (STRATAGENE). Following inactivation at 95°C for 10 minutes, 3 μ l of the resulting cDNA were used in a 100 μ l PCR reaction containing: 0.025 mM dNTPs (PROMEGA), 10mM DTT, 20U RNasin, 50U MMLV reverse transcriptase and 5 μ l of 10x reaction buffer (STRATAGENE).

35 Following inactivation at 95°C for 10 minutes, 3 μ l of the resulting cDNA were used in a 100 μ l PCR

-46-

reaction containing: 0.025mM dNTPs (PROMEGA), 0.5 μ M of PP2C α sense primer 5'-GAAGTAGTCGACACCTGT-3' (SEQ ID No:6), 0.5 μ M of PP2C α anti-sense primer 5'-GCTGGAGTCTGATTACAAAC-3' (SEQ ID No:5), 10x reaction buffer, 2.5mM MgCl₂ and 2.5u of ABTaq polymerase (Advanced BioTechnology). Thirty-five cycles of PCR were performed in the following conditions: 1 minute at 94°C, 1 minute at 60°C, 1 minute at 72°C, followed by 72°C for 10 minutes. The same cDNAs were used as templates for parallel PCR reactions performed in the presence of β -actin primers 5'GTTTGAGACCTTCAACACCCC-3' (SEQ ID No:7) and 5'GTGGCCATCTCTTGCTCGAAGTC-3' (SEQ ID No:8), in the same PCR reaction mixtures. Aliquots were taken after 20, 25, 30 and 35 cycles and analyzed by gel electrophoresis.

Results

The use of PP2C α specific oligonucleotide primers, generated RT-PCR products with the expected size of 480bp. The RT-PCR reactions demonstrated that in 7 out of 8 samples, the level of PP2C α mRNA were significantly higher in normal colon tissues than the levels obtained in the adjacent tumor colonic tissues. (Figure 7). The level of PP2C α expression in CHE cell line was higher than in the CO60 cell line (Figure 8).

EXAMPLE 6

PRODUCTION OF VECTORS AND TRANSFORMED CELLS HARBORING THE VECTOR

Expression of the PP2C α mRNA under the inducible tet promoter: Expression of the sense and antisense PP2C α mRNAs in mammalian cells is based on the system for tetracycline-regulated inducible gene expression as described by Gossen and Bujard [1992].

This system relies on constitutive expression of a tetracycline-controlled transactivator (tTA) fusion

protein which combines the tetracycline repressor with the activating domain of herpes simplex VP16. The tTA was constitutively expressed in rat fibroblasts and in HeLa cells. In these two cell lines the tTA stimulates transcription from a minimal promoter derived from the human cytomegalovirus promoter and the tetracycline operator. Upon addition of tetracycline the stimulation of transcription by tTA is inhibited.

Clones were prepared by stable transfection of the two cell lines with expression vectors that contain the PP2C α mRNA in the sense and in the antisense orientation, under the control of the tTA-dependent promoter.

Construction of expression vectors: The construction of the plasmids used as expression vectors included the following steps:

1. Preparation of the DNA fragment coding for the rat PP2C α mRNA.
2. Cloning of the rat PP2C α cDNA into the tTA containing plasmid.
3. Verification of the plasmids by restriction map analysis.
4. DNA sequence analysis of the PP2C α cDNA insert.

Preparation of the DNA fragment coding for the rat PP2C α mRNA: The DNA fragment coding for the rat PP2C α mRNA was prepared by thermal cycling amplification. The template for the amplification reaction was the insert of plasmid skPP2C (PP2C α cDNA cloned into the sk BLUESCRIPT plasmid).

The upper primer used in the amplification reaction contains the sequence coding for the first six amino acids of the rat PP2C α (Met Gly Ala Phe Leu Asp; SEQ ID No:9). The sequence of the upper primer is the following: 5' CGGGATCCGC ATGGGAGCAT TTTAGAC 3' (SEQ ID No:10).

The lower primer used in the amplification reaction contains the sequence coding for the last five amino acids and the stop codon of the rat PP2C α (Thr Asp Asp Met Trp ***; SEQ ID No:11). The sequence of
5 the lower primer is the following: 5' CGCGGATCCT TACCACATAT CATCAGT 3' (SEQ ID No:12).

The ends of the DNA fragments were modified by introduction of *Bam*HI restriction sites at both ends.
Cloning of the rat PP2C α cDNA into the tTA containing
10 plasmid: Following amplification of the rat PP2C α cDNA, the DNA fragment was cleaved with restriction enzyme *Bam*HI and cloned into plasmid pUHD10-3 [Gossen and Bujard, 1992] downstream from the tetracycline responsive promoter.

15 Verification of the plasmids by restriction map analysis: The orientation of the cDNA insert with respect to the promoter was determined by restriction map analysis. Plasmids that contain the cDNA in the sense orientation (pYM001) and in the antisense
20 orientation (pYM002) were selected (Figure 9).

DNA sequence analysis of the PP2C α cDNA insert: The sequence of the DNA insert of plasmid pYM001 was determined by automatic DNA sequence analysis. The primers used for sequencing analysis were the same as
25 the one used for cloning. The results of this analysis show that the sequence of the cloned fragment is identical to that of rat PP2C α and that no mutation was introduced during the amplification reaction.

Transfection: Plasmids pYM001 and pYM002 were
30 introduced in the rat fibroblast and in the HeLa cell lines which constitutively express the tTA, by CaPO₄ coprecipitation with plasmid pBSpac. Plasmid pBSpac contains a genetic selective marker, that confers puromycin resistance.

35 Twenty-four to forty-eight hours following transfection, cells were passaged 1:10 and grown in

-49-

selective medium. The selective medium for HeLa and for the rat fibroblast cell lines contained 0.3 ($\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$, neuromycin respectively. After two to three weeks clones were isolated, grown to confluence in 24 wells culture plates, transferred to 10 cm dish, grown to 70-90% confluence and frozen in 90% fetal calf serum 10% DMSO. In these clones following the removal of Tet we observed induction in PP2C α mRNA in clones harboring pYM001 and reduction in the endogenous mRNA of PP2C α in a clone harboring the antisense plasmid pYM002.

EXAMPLE 7

Role of integrated AAV in the modulation of PP2C α expression

This work does not identify the precise site of AAV integration within the gene. Additionally, the data does not provide the exact site for the human integration site which is also located on the same chromosomal region 19q13.3 but not within the 106kb cosmid. Applicants hypothesize that based on the results with RNA and the specific protein that the AAV integration site is either in the gene coding for pp2c α or its regulatory region is in some way associated with PP2C α . For example, rep might interact with pp2c α protein and the AAV genome linked to rep and PP2C α might be associated with the regulatory region of PP2C α .

In stable cell lines, SV40 amplification was suppressed by infection with recombinant AAV/neo virus and Applicants were not able to detect the expression of rep or the sequences coding for rep and, therefore, Applicants searched for a sequence with suppressing activity. This sequence was present in all the AAV harboring cells whether they are of Chinese hamster or mouse origin.

-50-

A functional correlation between the integrated AAV sequences and the modulation of PP2C α activity in the cells harboring the AAV genome was not demonstrated yet, however, it is clear that as a result of the AAV integration, there was an alteration in the transformed characteristics as described herein.

Based on the studies described below, it seems feasible that in addition to the site specific integration which occurs in the vicinity of PP2C α , the AAV sequences can have some importance in the alteration of the transformed phenotype.

The integrated AAV in SV40 transformed Chinese hamster cells (line 9-3 and other cell lines) is responsible for the suppression of the carcinogen induced SV40 amplification. The viral element responsible for the suppression of SV40 amplification (silencer, SEQ ID No:13) was defined using a transient assay for SV40 replication [Yang et al, 1995] to demonstrate that the AAV rep protein is responsible for the suppression of SV40 replication. This assay is based on transfection of the human kidney cell line 293 with an SV40 vector containing the coding region for T antigen and the viral origin of replication and cotransfection with several different constructs derived from the vicinity of AAV integration in 9-3, SV40 transformed Chinese hamster embryo cells containing an integrated AAV and DA357 (a mouse cell line harboring the integrated AAV in which the transformed phenotype was altered following AAV integration).

Using different constructs, Applicants succeeded to define the minimal AAV element conferring the suppression. This element is comprised of 64 nucleotides from the AAV genome (nucleotides 125-189).

ACTCCATCACTAGGGGTTCTGGAGGGGTG
GAGTCGTGACGTGAATTACGTCATAGGGTTAGGG

-51-

This element was termed SV40 silencer (SEQ ID No:13) though in an alternative embodiment only 21 nucleotides, 125-145, (SEQ ID No:14) are responsible.

5 The replication of the SV40 vector in 293 cells results in DNA which is not methylated and, therefore, is cleaved by *DpnII*, an enzyme that cleaves only unmethylated DNA. The *DpnII* digested DNA was separated on a gel, blotted and hybridized with SV40 probe. The results are shown in Figure 12.

10 The blot provides the following:

Lane 1: Cotransfection with pSK1 and pAV2 (a plasmid containing the whole AAV genome and expressing the rep protein). Note that SV40 replication is suppressed.

15 Lane 2: Transfection with the SV40 plasmid pSK1 SV40 replicates. Replication of the SV40 template is observed.

Lane 3: Cotransfection of pSVK1 and a plasmid harboring 138bp from the AAV genome (nucleotides 125-263). There was a suppression of SV40 replication by this element.

20 Lane 4: Cotransfection of pSVK1 with pSL9-6 (non AAV DNA sequences).

Thus SV40 replication in 293 cells was suppressed by rep and by the silencer element.

25 Similarly SV40 replication was suppressed when the cells were transfected by a plasmid containing only 125-145 (the SV40 mini-silencer, SEQ ID No:14).

5' A₁₂₄C₁₂₅TCCCATCACTAGGGGTTTCCT₁₄₅.

30 In control experiments in which Applicants transfected with other sequences derived from the integrated AAV and cellular sequences spanning the integrated AAV, such as pSL9-6 and others (see Figure 12, lane 4) no repression of SV40 DNA replication was detected.

35 Using a λ clone containing the integrated AAV and the flanking cellular sequences from the mouse DA3J7 cell line similar suppression of SV40 replication was

-52-

observed. This clone contains the 64 nucleotides comprising the silencer region.

Note that suppression of SV40 replication can be obtained in 293 cells by Rep expression and by the 64bp
5 silencer element in a transient assay.

Revertants which have lost the integrated AAV regained the capability to amplify SV40. In one revertant line, C9-3-2, applicant showed that the
10 revertants lost the whole chromosome containing the integrated AAV. Applicants showed this by FISH, the disappearance of a very well characteristic abnormal chromosome into which AAV integrated.

A hypothesis for the above observations can be made, but it is not to be construed as limiting the
15 present invention to this one mode of action. Applicants propose that the Rep protein [Heilbronn, Schlehofer et al, 1983; Kleinschmidt et al, 1995; Yang et al, 1995] and the silencer element can suppress SV40 replication by interaction with a similar protein or
20 element directly or indirectly, possibly PP2C α

It is possible that the 21 bp (mini-silencer) from AAV genome modulates PP2C α activity as well by interaction and activation of a control region. Alternatively, the silencer can act as a dominant
25 negative element interacting directly and/or indirectly with proteins associated with the replication of SV40. PP2C can regulate the action of such proteins by dephosphorylation. An example for such interplay can be the DNA polymerase α primase. It is possible that
30 the rep protein is directly also involved in such interactions.

It appears that dephosphorylate DNA polymerase α primase is responsible for the initiation of SV40 DNA replication during the carcinogen induced amplification
35 in CO60 cells. Moreover that this phosphorylate - dephosphorylate process are controlled by the cell

-53-

cycle. Thus PP2C α can modulate the activity of the DNA polymerase α primase depletion of PP2C α due to its binding to rep directly or indirectly might lead to aberrant phosphorylation of the DNA polymerase α primase and to its failure to initiate SV40 DNA replication.

EXAMPLE 8

THERE ARE MORE pp2c α PROTEINS THAN THE 42kd

10 Liver extracts were immunoprecipitated with the different monoclonal antibodies raised against rpp2c α (see Example 3 herein above). The precipitates were divided into two aliquots which were separated on 12% PAGE and blotted. Each set of immunoprecipitates was
15 challenged with the following polyclonal antibodies:
(1) 351 - which recognizes both α and β
(2) 801 - specific to pp2c α

As demonstrated in Figure 10 upon reaction with antibody 351 several bands migrating in the position of
20 40-43kd were detected. Monoclonal antibodies 9F11, 9F4 and 1D5 displayed a very similar picture of 2-3 strong bands and 1-2 faint bands. Monoclonal antibody 2A3 precipitated only the faint bands.

The second part of the blot was reacted with the α
25 specific antibody 801. In the position of ~40-43kd, two bands were visible with all four monoclonal antibodies. These bands are probably those which were detected by monoclonal 2A3. Thus, monoclonal antibody 2A3 is specific for pp2c α .

30 In addition, two additional bands migrating in the position of 75kb and greater than 150kd were detected. These proteins are more abundant in liver than the 40-43kd protein. Since all eight monoclonal antibodies recognized these proteins and since the 801 polyclonal
35 antibody reacted as well, it is clear that these two large proteins share several epitopes with pp2c α

-54-

suggesting that they are the product of the same gene but result from alternative splicing.

5 A weak reaction with the 75kd protein is detected with polyclonal antibody 351 when reacted with the immunoprecipitate. However, upon direct immunoblotting of total liver extract, it seems that 75kd was present and a faint reaction could be detected against the higher molecular weight protein.

10 Several additional higher molecular weight proteins were also detected with polyclonal antibody 351. These proteins did not interact with polyclonal antibody 801, which is specific to pp2c α . These results suggest that other forms of α exist and have different molecular weights.

15 Northern blot analysis (Figure 13) of mRNA derived from several tissues displayed several bands of RNA which hybridized with probes derived from the 5'UTR of pp2c α or with the entire PP2C α cDNA probe. The RNA was extracted from different tissues and different sizes of
20 RNA appeared in these tissues.

EXAMPLE 9

pp2c α REGULATES mRNA SYNTHESIS

25 Table 6 summarizes the protein or RNA sequence homology that was found for the 10 amino acid pp2c α carboxy terminal peptide: NDDTDSASTD (SEQ ID No:1). This peptide was used to raise polyclonal antibody 801 as described herein above.

30 The carboxy cellular domain (CTD) of the RNA polymerase II fused to GST and bound the fusion complex to sepharose glutathion beads and mixed with HeLa cell extract. Following PAGE and blot the proteins bound to the carboxy terminal domain (CTD) of the RNA polymerase
35 bound also to pp2c α . Both 801 and 2A3 were used with the blot. The size of the associated pp2c α was

-55-

approximately 43kD. Thus, RNA polymerase II is associated with pp2c α .

In a second experiment, extracts from tumor cells, Hepatoma and HeLA, were assayed as above. Binding was
5 observed to GST-CTD only in the tumor extracts while in cell extracts from normal heptocytes such activity was not detected.

Based on the studies [Chambers and Dahmus, 1994] which demonstrated that the polymerase α CTD domain can
10 be dephosphorylated by a phosphatase similar in its properties to PP2C α (but not PP2C α) applicant proposes that pp2c α dephosphorylates RNA polymerase II and thus regulates the initiation of mRNA synthesis on specific messenger. This peptide can be used to control and
15 regulate transcription facilitated by other factors.

EXAMPLE 10

FURTHER SEQUENCES

Two λ clones containing the AAV integration site
20 were prepared. (1) One was derived from the chinese hamster cell CO60 designated λ SL9-1 (schematic diagram in Figure 3; SEQ ID Nos:15 and 16). Parts were sequences as indicated in Figure 3. A further sequence AN8T7 (SEQ ID No:18) was derived from plasmid pSL9-8
25 (Figure 3). (2) The second λ phage was cloned from the cell line DA3J7 and was not mapped. Portions were subcloned to plasmids and part sequenced as set forth in 5h-1 (SEQ ID No:17) A sequence comparison shows that 5h-1 is homologous to AN8T7. This region of
30 comparison was also homologous to the cosmid MMDA 23,467-23715. Throughout this application, various publications are referenced by citation and patents by patent number. Full citations for the publications are listed below. The disclosures of these publications in
35 their entireties are hereby incorporated by reference

-56-

into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that
5 the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the
10 above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

Table 1. Plating efficiency of the DA₃J clones compared with that of the parental DA₃ cell line

No. of cells plated	No. of outgrowing colonies (mean \pm SD), % of plating efficiency			
	DA ₃	DA ₃ J ₁	DA ₃ J ₂	DA ₃ J ₃
250	88 (6) 35%	53 (8) 21%	78 (11) 31%	43 (5) 17%
500	181 (36) 36%	103 (10) 20%	131 (20) 26%	82 (9) 17%

Semiconfluent cells derived from DA₃, DA₃J₁, DA₃J₂, and DA₃J₃ cell cultures were seeded at 250 and 500 cells onto 9-cm plastic petri dishes. Cultures were grown for 7 days. Cells were then fixed and stained with Gimsa. The mean number of growing colonies from two experiments was determined. Each experiment was performed in triplicates.

Table 2. UV sensitivity of the DA₃J clones compared with that of the parental DA₃ cell line

J/m ²	No. of outgrowing colonies (mean \pm SD), % of survival							
	DA ₃		DA ₃ J ₁		DA ₃ J ₂		DA ₃ J ₃	
0	91 (4)	100%	59 (20)	100%	79 (6)	100%	36 (7)	100%
2.5	86 (9)	95%	54 (9)	91%	85 (8)	100%	36 (4)	100%
5	62 (5)	68%	25 (30)	42%	39 (13)	49%	11 (11)	30%
10	22 (6)	24%	12 (2)	20%	19 (4)	24%	4 (2)	11%
20	0 (0)	0%	0 (0)	0%	2 (2)	2%	0 (0)	0%

Semiconfluently growing cells from DA₃, DA₃J₁, DA₃J₂, and DA₃J₃ cell cultures were seeded at 250 cells onto 9-cm plastic petri dishes. After 48 hours the cultures were irradiated with UV light, and then incubated for 7 days. The mean number of growing colonies was determined. The experiment was performed in triplicates.

Table 3A

File: Shu..008

Acquisition Date: 10-May-95

Total Events: 7000

X Parameter: FL2-A (Linear)

Marker	Events	% Total	Mean	CV
All	7000	100.00	384.11	48.19
G0/G1	3355	47.93	394.88	14.49
S	943	13.47	569.28	7.41
G2+M	668	9.54	727.76	8.06
Ap.	1485	21.21	132.36	36.21

TABLE 3B

File: Shu..010

Acquisition Date: 10-May-95

Total Events: 7000

X Parameter: FL2-A (Linear)

Marker	Events	% Total	Mean	CV
All	7000	100.00	382.46	51.58
G0/G1	3019	43.13	393.54	14.79
S	938	13.40	574.35	7.10
G2+M	798	11.40	737.43	8.24
Ap.	1684	24.06	133.69	36.68

Table 4:
Characterization of Monoclonal Antibodies

Antibody number	Antibody capture (1)		Immuno-blotting (2)	Immuno- (3) precipitation
	PP2C	PET		
1D5	+ 1/8	-	+++	+++
2A3	+ 1/32	-	+++	α -specific
2H8	+ 1/16	+ 1/8	not tested	+++
9F4	+ 1/10	-	+++	+++
9F11/169	-	-	nonspecific	+++
9F11/53	+ 1/10	-	+++	+++
10C6	+ 1/10	+ 1/10	++	+++
10F8	+ 1/1	-	+	+++

(1) Antibody capture assay was performed on two antigens: purified rpp2c - to identify anti pp2c antibodies, and a protein extract from cells containing the pET expression vector without the pp2c insert - to identify non-specific antibodies. Results are expressed as + or - and the number indicates the antibody dilution giving the highest result.

(2) Immunoblotting tested on rpp2c and on liver extracts separated on 12% SDS-PAGE.

(3) Immunoprecipitation. Monoclonal antibodies were used to precipitate proteins from a liver extract. The proteins were separated on 12% SDS-PAGE and detected by affinity purified rabbit polyclonal antibodies - 801 which is α -specific and 351 which identifies pp2c α and pp2c β .

- 60 -

TABLE 5

Homology between CHINT (SEQ ID No:19) and
a sequence from human DNA from cosmid DNA
MMDB (SEQ ID No:20) containing the 5' end
of the PP2C RNA

LOCUS HUMMMDBC 68505 bp ds-DNA PRI 09-APR-1992
DEFINITION Human DNA from cosmid DNA MMDB (f10080) and MMDC (f13544) from
chromosome 19q13.3 (obtained by automated sequence analysis).
ACCESSION M89651 M77823 M77824 M77825
KEYWORDS
SOURCE Homo sapiens (library: Lawrist5 vector library of A.V. Carrano)

SCORES Init1: 61 Initn: 150 Opt: 82
58.3% identity in 103 bp overlap

```
      20      30      40      50      60      70
int.li TAGTGCCGGTCAAGGAACTGAACGTGCGATTCCGGGACAGGCTACCCACTCCGATCCCAG
      ||      |      |      |      |      |      |      |      |
hummdb CCTCACCTCCGCCCTGTTTCGTCCAGGTCCTCCGGGTCAGGCTACCCCGTCGCCGCCA-
      57710      57720      57730      57740      57750      57760

      80      90     100     110     120     130
int.li GAGAAGTTGTCATGGTGAGGGCCACCCTAGGTCTCTGCCCTGCTGTGTCCCCCATCTTA
      |||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||
hummdb GAG-CGCGGGGGAGGGGAGAGCTTCCTTTGTCTCCTATGCCTCCT---CCCCCATCCCG
      57770      57780      57790      57800      57810      57820

     140     150     160     170     180     190
int.li CCCATCCAGTAGGATCTAGAGGCTGTGCGCCCCCTTGTGGAATGCACAGAAGTCACAAGCG
      ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||
hummdb GCTCTCCTGCGGGCAAGCGCCGAGGGGACACCGGGGAGTACCCACCTGAACCTCTGGGG
      57830      57840      57850      57860      57870      57880
```

TABLE 6

Proteins or RNA Sequences Homologous to NDDTDSASTD

Peptide sequence

- #1
- 1) To the different PP2C α protein and mRNAs.
 - 2) To rattus norvegian neurona. pentroxin precursor mRNA.
 - 3) To *Xenopus* transcription factor IIIA.
 - 4) To Human DNA/RNA binding protein mRNA.
 - 5) To Human transcription factor IIIA.

and to other additional proteins to a lesser extent.

Homologues on the RNA level

- #2
- 1) PP2C α mRNAs
 - 2) Human mRNA homologue to *Xenopus* transcription factor IIIA.
 - 3) Human DNA/RNA binding protein mRNA.
 - 4) Human transcription factor IIIA.
- #3
- 1) Different forms of PP2C α
 - 2) *C. elegans* cosmid coding for DNA directed RNA polymerase sigma chain.
 - 3) Potato mRNA for pyruvate kinase.
 - 4) *C. elegans* cosmid.
 - 5) Ictanurid herpes virus
DNA polymerase helicase
 - 6) *A. thabana* mRNA for UIsnRNA specific protein UIA.
 - 7) *Ascaris lumbricoides* small nuclear RNA (snRNA UI-1 UI-2 UI-3 U-I genes).

REFERENCES

Aladjem and Lavi, 1992. The mechanism of carcinogen-induced DNA amplification: In-vivo and in-vitro studies. *Mutation Res.* 276:339-344.

Atchison et al., 1965. Adenovirus-associated defective virus particles. *Science* 149:754-756.

Atherton-Fessler et al. (1993) Reversible tyrosine phosphorylation and cell cycle control. *Semin. Cell. Biol.*, 4(6):433-42.

Bantel-Schaal and Zur Hausen (1988a) Adeno-associated viruses inhibit SV40 DNA amplification and replication of herpes simplex virus in SV40-transformed hamster cells. *Virology*, 164:64-74.

Bantel-Schaal and Zur Hausen (1988b) Dissociation of carcinogen-induced SV40-DNA-amplification and amplification of AAV DNA in a Chinese hamster cell line. *Virology*, 166:113-122.

Berns, 1990. Parvovirus replication. *Microbiol. Rev.* 54:316-332.

Brown, et al. (1996) A defect in nurturing in mice lacking the immediate early gene *fosB*. *Cell*, Vol 86, pp. 297-309.

Burke and Olson, 1991. Preparation of Clone Libraries in Yeast Artificial-Chromosome Vectors" in *Methods in Enzymology*, Vol. 194, "Guide to Yeast Genetics and Molecular Biology", eds. C. Guthrie and G. Fink, Academic Press, Inc., Chap. 17, pp. 251-270.

Burstyn, 1993. Suppression of SV40 DNA amplification by Adeno Associated virus. M.Sc. Thesis. Tel Aviv University.

Capecchi, 1989. Altering the genome by homologous recombination. *Science* 244:1288-1292.

Casto and Goodheart, 1972. Inhibition of adenovirus transformation in vitro by AAV-1. *Proc. Soc. Exp. Biol. Med.* 140:72-78.

Cech (1986) "RNA as an Enzyme", *Scientific American*, 255:64-75

Cech (1990) "Self-Splicing of Group I Introns", *Annu. Rev. Biochem.* 59:543-568

Chambers and Dahmus, 1994. Purification and characterization of a phosphatase from HeLa cells which dephosphorylates the c-terminal domain of RNA polymerase II. J. Biol. Chem. 269(42):26243-26248.

Chambers et al. (1995) The activity of COOH-terminal domain phosphatase is regulated by a docking site on RNA polymerase II and by the general transcription factors IIF and IIB. Journal of Biological Chemistry, 270(25):14962-14969.

Cheung et al, 1980. Integration of the Adeno-associated virus genome into cellular DNA in latently infected human Detroit 6 cells. J. Virol. 33:739-748.

Cohen, 1989. The structure and regulation of protein phosphatases. Annu. Rev. Biochem. 58:453-508.

Cohen, P. 1991. classification of protein-serine/threonine phosphatases: identification and quantitation in cell extracts. Methods. Enzymol. 201:389-399.

Cukor, et al, 1984. Biology of adeno-associated virus. In: Berns, K.I. (Ed.) The Parvoviruses. Plenum Press, New York, pp. 33-66.

Davies et al., 1992. Targeted alterations in yeast artificial chromosomes for inter-species gene transfer. Nucleic Acids Research, 20(11):2693-2698.

Dickinson et al., 1993. High frequency gene targeting using insertional vectors", Human Molecular Genetics, 2(8):1299-1302.

de la Maza and Carter, 1981. Inhibition of Adenovirus oncogenicity by Adeno-associated virus DNA. J. Natl. Cancer Inst. 67:1323-1326.

Eden and Cedar (1994) Role of DNA methylation in the regulation of transcription. Curr. Opin. Genet. Dev., 4(2): 255-9.

Fodor et al, 1993. Multiplexed biochemical assays with biological chips, Nature 364:555-556.

Fukunaga, et al, 1993. Dephosphorylation of autophosphorylated Ca²⁺/calmodulin-dependent protein kinase II by protein phosphatase 2C. J. Biol. Chem. 268:133-137.

Gavrieli, et al, 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell. Biol.* 119:439-501.

Georg-Fries, et al, 1984. Analysis of proteins, helper dependence, and seroepidemiology of a new human parvovirus. *Virology* 134:64-71.

Gilboa, et al, 1986. Transfer and expression of cloned genes using retroviral vectors. *BioTechniques* 4(6):504-512.

Gossen and Bujard, 1992. *Proc. Natl. Acad. Sci. USA* 89, 5547-5551.

Hampel et al. (1993) "The Hairpin Ribozyme", *Methods: A Companion to Methods in Enzymology* 5:37-42

Hermonat, 1989. The Adeno-associated virus rep78 gene inhibits cellular transformation induced by Bovine Papillomavirus. *Virology*. 172:253-261.

Hermonat, 1994. Down regulation of the human c-fos and c-myc proto-oncogene promoters by Adeno-associated virus rep78. *Cancer Lett.* 81:129-136.

Huxley et al., 1991. The human HPRT gene on a yeast artificial chromosome is functional when transferred to mouse cells by cell fusion. *Genomics*, 9:742-750 (1991).

Inouye, 1988. Antisense RNA: its functions and applications in gene regulation - a review. *Gene*. 72:25-34.

Jakobovits et al., 1993. Germ-line transmission and expression of a human-derived yeast artificial chromosome, *Nature*, 362:255-261.

Justement et al. (1994) Regulation of B-cell activation by CD45: a question of mechanism. *Immunol. Today*, 15(9):399-406.

Kafri, et al, 1992. Developmental pattern of gene-specific DNA methylation in mouse embryo and germ line. *Genes Dev.* 6:705-714.

Katz and Carter, 1986. Effect of adeno-associated virus on transformation of NIH 3T3 cells by ras gene and on tumorigenicity of an NIH 3T3 transformed cell line.

Kawasaki, 1990. Amplification of RNA. In: PCR protocols: A Guide to Methods and Applications, Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. Academic Press, pp21-27.

Kirschstein, et al, 1968. Inhibition of adenovirus 12 oncogenicity by adeno-associated virus. Proc. Soc. Exp. Biol. Med. 128:670-673.

Kleinschmidt et al. (1995) Sequence elements of the adeno-associated virus rep gene required for suppression of herpes-simplex-virus-induced DNA amplification. Virology, 206:254-262.

Lamb et al., 1993. Introduction and expression of the 400 kilobase precursor amyloid protein gene in transgenic mice", Nature Genetics, 5:22-29.

Lau and Baylink (1993) Phosphotyrosy: protein phosphatases: potential regulators of cell proliferation and differentiation. Crit. Rev. Oncog., 4(4):451-71.

Lavi, 1981. Carcinogen mediated amplification of viral DNA sequences in SV40 transformed Chinese hamster embryo cells. Proc. Natl. Acad. Sci. USA 78:6144-6148.

Leonard and Berns, 1994. Adeno-associated viruses type 2: a latent life cycle. Proc. Natl. Acad. Sci. USA. 48:29-52.

Liang and Pardee, 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257:967-971.

Lichter, et al., 1990. High-resolution mapping of human chromosome 11 by in situ hybridization with cosmid clones. Science 247:64-69.

Mann et al., 1992. Mammalian protein serine/threonine phosphatase 2C: cDNA cloning and comparative analysis of amino acid sequences. Biochim. Biophys. Acta 1130:100-104.

Martin-Gallardo et al. (1992) Automated DNA sequencing and analysis of 106 kilobases from human chromosome 19q13.3. Nature Genetics, Vol. 1, pp. 34-39.

Mayor, et al, 1973. Influence of adeno-associated satellite virus on adenovirus-induced tumors in hamsters. Nature New Biol. 261:44-46.

Mayor, et al, 1976. Antibodies to adeno-associated satellite virus and herpes simplex in sera from cancer patients and normal adults. *Am. J. Obstet. Gynecol.* 126:100-104.

Martin-Gallardo, et al, 1992. Automated DNA sequencing and analysis of 106 kilobases from human chromosome 19q13.3. *Nature Genetics.* 1:34-39.

McClelland, et al, 1995. RNA fingerprinting and differential display using arbitrarily primed PCR. *TIG.* 11:242-246.

McGowan and Cohen (1987) Identification of two isoenzymes of protein phosphatase 2C in both rabbit skeletal muscle and liver. *Eur. J. Biochem.*, 166:713-722.

McGowan and Cohen, 1988. Protein phosphatase-2C from rabbit skeletal muscle and liver: an Mg 2+ dependent enzyme. *Methods Enz.* 159:416-426.

Nishikawa et al. (1995) Up-regulation of protein serine/threonine phosphatase type 2C during 1 alpha, 25-dihydroxyvitamin D3-induced monocytic differentiation of leukemic HL-60 cells. *FEBS Lett.*, 375:299-303.

Ohishi, S. et al., *Biochem. Intl.* 28:345-351, 1992

Orita M, et al. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci USA* 1989; 86:2766-2770

Ostrove, et al, 1981. Inhibition of adenovirus-transformed cell oncogenicity by adeno-associated virus. *Virology* 113:521-533.

Pease et al., 1994. Light-generated oligonucleotide arrays for rapid DNA sequence analysis. *Proc. Natl. Acad. Sci. USA* 91(11):5022-5026.

Robinson, et al, 1994. TPD1 of *Saccharomyces cerevisiae* encodes a protein phosphatase 2C-like activity implicated in tRNA splicing and cell separation. *Mol. Cell. Biol.* 14:3634-3645.

Rommelaere and Tattersall, 1990. Tijssen P. (ed.), *Handbook of Parvoviruses*. CRC, Boca Raton, pp. 41- 85.

Rothstein, 1991. "Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast" in *Methods in Enzymology*, Vol. 194, "Guide to Yeast Genetics and

Molecular Biology", eds. C. Guthrie and G. Fink, Academic Press, Inc., Chap. 19, pp. 281-301.

Roth et al., 1990. Yeast alpha 2 repressor positions nucleosomes in TRP1/ARS1 chromatin. *Mol. Cell. Biol.*, 10:2247-2260.

Saadat, et al. (1994) Gene expression of protein phosphatases in rat ascites hepatoma cell lines. Cancer Detection and Prevention, 18(2):115-122.

Sato and Mayor, 1979. Adenovirus-associated virus polypeptides synthesized in cells coinfecting with either adenovirus or herpesvirus. *Virology* 93:237-245.

Schedl et al., 1993. A yeast artificial chromosome covering the tyrosinase gene confers copy number-dependent expression in transgenic mice, *Nature*, 362:258-261.

Schlehofer et al. (1983) Inhibition of initiator-induced SV40 gene amplification in SV40-transformed Chinese hamster cells by infection with a defective parvovirus. Int. J. Cancer, 32:591-595.

Schlehofer (1994) The tumor suppressive properties of adeno-associated viruses. Mutation Research, 305:303-313.

Schlehofer, et al, 1986. Vaccinia virus, herpes simplex virus, and carcinogens induce DNA amplification in human cell line and support replication of helper virus dependent parvovirus. *Virology*. 152:110-117.

Schlehofer and zur Hausen, 1982. Induction of mutations within the host cell genome by partially inactivated herpes simplex virus type 1. *Virology* 122:471-475.

Shiozaki and Russell (1995a) Counteractive roles of protein phosphatase 2C (PP2C) and a MAP kinase kinase homolog in the osmoregulation of fission yeast. The EMBO Journal, Vol. 14, No. 3, pp. 492-502.

Shiozaki and Russell (1995b) Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. Nature, Vol. 378, pp. 739-743.

Shiozaki, et al, 1994. Protein phosphatase 2C, encoded by *ptc1+*, is important in the heat shock response of *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* 14:3742-3751.

Siegl, et al, 1985. Characteristics and taxonomy of Parvoviridae. Intervirology 23:61-73.

Soderling (1993) Protein kinases and phosphatases: regulation by autoinhibitory domains. Biotechnol. Appl. Biochem., 19(Pt.2):185-200.

Sotomayor, et al, 1991. Role of the tumor derived cytokines on the immune system of mice bearing a mammary adenocarcinoma. J. Immunol. 147:2816-2823.

Sprecher-Goldberger, et al, 1971. Complement-fixation antibodies to adeno-associated viruses, adenoviruses, cytomegaloviruses and herpes simplex viruses in patients with tumors and in control individuals. Am. J. Epidemiol. 24:351-358.

Sullivan (1994) "Development of Ribozymes for Gene Therapy", J. Investigative Dermatology (Suppl) 103:95S

Strauss et al., 1993. Germ line transmission of a yeast artificial chromosome spanning the murine α_1 (I) collagen locus, Science, 259:1904-1907.

Tamura et al. (1989) Molecular cloning of rat type 2C (IA) protein phosphatase mRNA. Proc. Natl. Acad. Sci. USA, Vol. 86, pp. 1796-1800.

Taylor, 1980. J. Histochem. Cytochem. 28:1021.

Tratschin, 1985. Adeno associated virus vector for high frequency of integration, expression, and rescue of genes in mammalian cells. Mol. Cell. Biol. 5:3251-3260.

Vindelov, et al, 1983. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry 3:323-327.

Walz and Schlehofer 1992. Modification of some biological properties of Hela cells containing Adeno associated virus DNA integrated into chromosome 17. J. Virol. 66:2990-3002.

Wang et al. (1996) A Mg(2+)-dependent, Ca(2+)-inhibitable serine/threonine protein phosphatase from bovine brain. The Journal of Biological Chemistry, Vol. 270. No. 43, pp. 25607-25612.

Weinberg, R. (1996) E2F and cell proliferation: a world turned upside down. Cell, Vol. 85, pp 457-458.

Wenk and Mieskes (1995) Cytosolic and nuclear localization of protein phosphatase 2C β 1 in COS and BHK cells. Eur. J. Cell Biology, 68:377-386.

Wera and Hemmings (1995) Serine/threonine protein phosphatases. Biochem. J., 311:17-29.

Wijsman, et al, 1993. A new method to detect apoptosis in paraffin sections: in-situ end-labeling of fragmented DNA. J. Histochem. Cytochem. 41:7-12.

Winocour, et al, 1992. Modulation of the cellular phenotype by integrated Adeno-associated virus. Virology. 190:316-329.

Yakobson, et al, 1989. Replication of Adeno-associated virus in cells irradiated with UV light at 254 nm. J. Virol. 63:1023-1030.

Yang et al. (1995) Inhibition of cellular and SV40 DNA replication by the adeno-associated virus rep proteins. Virology, 207:246-250.

Yakura (1994) The role of protein tyrosine phosphatases in lymphocyte activation and differentiation. Crit. Rev. Immunol., 14(3-4):311-36.

Young and Mayor, 1979a. Adeno-associated virus - an extreme state of viral defectiveness. Prog. Med. Virol. 25:113-132.

Young and Mayor, 1979b. Studies on the defectiveness of adeno-associated virus (AAV). 1. Effects of phosphonoacetic acid and 2-deoxy-D-glucose on the replication of AAV. Virology 94:323-341.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Lavi, Sara
- (ii) TITLE OF INVENTION: MANIPULATION AND DETECTION OF PROTEIN PHOSPHATASE 2C -PP2CALPHA- EXPRESSION IN TUMOR CELLS FOR CANCER THERAPY, PREVENTION AND DETECTION
- (iii) NUMBER OF SEQUENCES: 20
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Kohn & Associates
 - (B) STREET: 30500 Northwestern Hwy.
 - (C) CITY: Farmington Hills
 - (D) STATE: Michigan
 - (E) COUNTRY: US
 - (F) ZIP: 48334
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Kohn, Kenneth I.
 - (B) REGISTRATION NUMBER: 30,955
 - (C) REFERENCE/DOCKET NUMBER: 2290.00037
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (810) 539-5050
 - (B) TELEFAX: (810) 539-5055

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asn	Asp	Asp	Thr	Asp	Ser	Ala	Ser	Thr	Asp
1				5					10

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 amino acids

-71-

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Tyr	Lys	Asn	Asp	Asp	Thr	Asp	Ser	Thr	Ser	Thr	Asp	Asp	Met	Trp
1				5				10						15

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 9 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Pro	Asn	Lys	Asp	Asn	Asp	Gly	Gly	Ala
1				5				

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGGATCAAGT CATAATGGGA

20

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "Primer"

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
GCTGGAGTCT GATTACAAC

20

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
GAAGTAGTCG ACACCTGT

18

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
GTTTGAGACC TTCAACACCC C

21

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
GTGGCCATCT CTTGCTCGAA GTC

23

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid

-73-

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Gly Ala Phe Leu Asp
1 5

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGGGATCCGC ATGGGAGCAT TTTTAGAC

28

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 5 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Thr Asp Asp Met Trp
1 5

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGCGGATCCT TACCACATAT CATCAGT

27

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 64 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "Silencer Region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACTCCATCAC TAGGGGTTCC TGGAGGGGTG GAGTCGTGAC GTGAATTACG TCATAGGGTT 60
 AGGG 64

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "Mini-silencer region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ACTCCCATCA CTAGGGGTTC CT 22

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1573 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "35-3.seg (Figure 3)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AAGCTTGTCA AAATTACTAT TCAGTGTGAT TTTTAGTGGA TGAAACCTCA TGA CTAGTAT 60
 ATTATGACAT TAGCTTTGCG TAGTGAAGGC ACAAGCTGCT AAGTGGTTAG GGATGTATTT 120
 TGCCGTAGCC TGTATCACNC CAGGTCCTGG GCTCGGTTCC TAGCATTACA GGAAAAAGCA 180
 GGCGGTGGTT GACCTTTAAT GAATGGATTT TTCAATTTAG AAGTTGGTTT CATTTTAAAG 240
 AATTCAAAAA TGTTCCCAT AGCACTTTGT TTTGACATTG AGATCAGCTG CTAATTGAGG 300
 TCCAGTATAT ACTTAGAAAA CTGAGCGAAA CTTTGATGGA CACACACACA CACCCCTGTT 360
 GTTCATTTAA TAATTGAACT AAATAAATA CTGTTTAGTC ATCCACGTAA GCAAGAGGCC 420

TGTGTAAACA GTATTTGTAT TAGTAAAAAC TTTATAACAT AGTTACATAA TCAGCATCAT	480
TTTTTTTATG GACCTTATAG TTGGCTACTT CACTGGGTTT GTTATAATTT AATCAGACTC	540
CTAAATAGGT TAAATTTCTG AATTGCCTAC TTCAGTTTTG AAGAATTATT TTGTTTCATA	600
ATTTCCCATG CATATCTGGT AAATAATTCT GGATTGTTTC TAAAGGGGAG AGCAAGGTCT	660
CTTATGCAAA GTGAAAATCT AGATATGCTG TTTGTAAGAA TATAATAGTG ATAAAGTAGT	720
GTCCTTTTGC TCAGTGCCTC CATTCTTACC AGGCTGTGAC TGATCTTCAG TATTATTCAG	780
ACAGTCACTA TTAATATATC CGTTGCACAG TGGGGAAATT GAGGGAAGTT AGATAGGCAT	840
CGGGTATCTT AATCATAACT CACATATACC CAGCTGGCTA GTCAGCCTAG CTAAGACAGT	900
TCACACCCAG TTGAGGCAGC TTGCTGTTGG CCATTAGTAG GTAACCTAAT GGCTTGTTT	960
CTTCACTGGT AAGGTGGGGA TATAATAATG CCAATAATTG CATAATGATT AAAGACATTA	1020
ATATATTCCA TAAATTTTCC TGAATAGTGC TTAGCTGGTA CCCCTCCCCA CACATGCACC	1080
CCAGTCCAAT GTTCAGATGT TTACTTTGTT AAGCCCAGTT AATCCATTCC CCCTAATATC	1140
TTCTCCCACT TTGAAGAANG TTGAAGAATG TTGGGCTTGT TAGTTTAATT TTTTAAGAAG	1200
CATATCATGT TGCTTTTTTA AAACATGTTT CTTTGGGTTT TGGCTTCCCC TTTTGGAAG	1260
AATTCCAATT TACACTTATG GAAGAAAGCC ATTGTCCCCT CCAATTTCCC CCCCTGTCCC	1320
TTTCCAATAC AGCCCAACTC CCCATGTTTT GACTTCCTCC CCTGAACCAC CCCGTTCTCC	1380
TGTTTTTCCC TCCCCANAA AAAAAACCCA ATAATTTGAC TTTGGTAATT GAATTTCCCG	1440
CCNGTTAGGC NCCTGAATTG CCGAAATAAT TCCCCCGTGC NCCCNNGGANT TTTGGCACCC	1500
CCTGCCCCCTT AACCTGTTCT GCTGCCCCCC ATTTTAAAT GGCTTGCCGC NTTACNCCAA	1560
ANACTGCCTT TCC	1573

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2580 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "35-T7.seg (Figure 3)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTCGATCTCA CAAAGTCACA GAGCTCTTCG TTTCCCATGA CATCCCAGAT ACCATCACAT	60
GCAAGAATAA TGAAGTATC GTCCTCTTCA GACCTTTCAA TATCATGGAC TTCTGGCTCT	120
GGTGAGACGA GCTGCTCTGT GGGACCTTTT CCATGGACAC ATTTGTAATC GAAATCCCCA	180
AGGGCCCTTG ACACAGCCAG AGAGCCATTT ACACGCTGAA TCATCACAGA GCCCCCTGCA	240
TTCTGAATTC GTTCTTTTTC CAGCGGGTTA CTTGGTTTGT GGTCTTGTGT GAAGAAGTGA	300

ACTTTCCTGT TTCTACAAAG CAAACCTCTC GAGTCTCCAC AGTTAATGAA GTAAGTATGT	360
TTGGGGAGAA ATTAAGACCC CCACAGCTGT TTGACCCACT TCCTATCTGC ACCATGTTTT	420
CCTTCCTCCT GACATGACTC CTCATGTTGT TTCCATCAAT CTCCCAGAAA AACCTGTTCC	480
TGATCCCCAT TCCTTTACAT TTTCCACAG AAAGGTGCTC CCTGCAGAGC CTTTTAAAT	540
CCCTGGTTTA TTGGTGATGT TGATTCTNAA CAAATGCTCC ACAGCCAGTA TTTNGGCAAC	600
CTTGAAAAAC CAGCATGCCC ATCCATATAC AGCCAAGAAT GACCATGTTT TCCAGTTCCA	660
CTTTNGGCAA ACCCAATCCA CAGCCGTTNT GCGCATCCTC CCATTTCAAC TCCGCCAAC	720
CNTTGCNTGC TGCNTTAAGC CATATCGCAA CCCATCCCCC CTGCCCCCTG GGGCATTATG	780
CNTTTCATC TTTGGTTGTC TAAAATGCTC CCATTATGAC TTGATCCTCT AGGTCTGCAA	840
AGGAAGAGAA ATAAGAAAGT TAGTAACTGT CTTTGAAACA AAGCACACAT CCAACAGTCT	900
TTTTGAAGCA CCTACGAGAT ACAAGGAAAC GTAAAACTC ATAGGCTATA GCCATAAGCA	960
TTGTTCTACT GACTTGAAA ATGTAGAGAT TAATAAGAA GGGAAAGGCT GATCAAGTAC	1020
AGCTCAACCA GACAAGCAGC AGATGGAAC AAGTCACCAG GTAAAAGAGA GCTTGTTTGC	1080
CTCTCTGTGA TACCAAGGAG GCCCAGCAGT GACCATTAAC TTACATGAAC TAGGCAAGAT	1140
TTCAGGGTGC ATTCATCATA TGTAACCTCT CAATTAAGTT GTGTGTTGAT TAAAAAAAT	1200
AATTCATAGA AACATACAAG TATCTACTAC TTCAGGGAAC CTTAGCTAAG TACTCAGGAA	1260
TGTTGAGAGT TTGATTCCAT GCTATTTAGT TTTGTTTCTA CAACTAGATA CCTTTGGTAA	1320
AAATAAAAAG TAATTACTCA CACTGGTCCA AATTTTCAGT GCCTTGTCGA GGTCAATCTC	1380
TTTAGCTGGA ATTCCTGCC TCACCTCTTT ACCAACAGAA AAAAAATACA CCTGTTTCTA	1440
TCCTTTGAAA TCCAGTTCAA TTGTTCCCCC TTCCTCCAGA CTTTACAGTC CTTGAAAAAA	1500
ACAAGTTATT AACTACAGAA GTCAGCTTCC ATTTCCAGTT NGGAATGTTT TTTAATGAAC	1560
AATTTTATTG TTCNAAATCT NACNATATGA TAACTAANCN AATGGTAATA ATATTTTCAN	1620
CCCTGCCCTA TGGCCGCTNT TTTAATCCT NAAAAAATC NAAGGTCTAT TCCNCCNNC	1680
CTTGCCAATA CTTNACANCN CCAGTTCCCT GATCTGGAAT GGACCCACAA AGGTCAAGAC	1740
TTAGGTTANC CCTTGCTCAC AACTAAAGA AAATCTTAAA GGAGAACAGA ATACTGAAGA	1800
GAGAAATGAG GGTGAAGGAC AGTGTTTCAGG TGACGTTCTG AAACCAGGGG ACTAAANATA	1860
CCANAANTGG TGTTNCAGAC AGAAATGGTA TGGAATACTC CTTAGGAAAG AAATGACANN	1920
TNTTGTTCG CAGCAACCCC CNCACATGGC TTTCTCTTTT TCCTTCTGCT GATTAAGTGA	1980
TGCACNTGGT ANAAAAGTCA ACANACCCCT CCTCCACNCA GACTCCCACC GAGTACANNG	2040
GCCCATGTGC TCANTACACT CTGCCCCAAA CTCNNANNAT TCATTCNNCT CCCNTGTNA	2100
TTTATNAGGG CCTTCCCNCT CAGTTNTCTN ATCNCCAACG GANATTANCC TTCCANNAT	2160
TTACCCCCNN TTTGTACANC ACATNNTGGC NNGTGCCACN GTTANGCGTC GGCNTCCCTG	2220
TTNCACTNCA TCCCTCATCN TTAGGCCANG TTTGATTCTC CNGTGACANAN TTTCCGCANN	2280

ANCNTACCCC TTGCACNTC CATNTCTNNG GAANAACCTC CGGTTCTGAA TCTNCCCCNN	2340
TCCCGTCNCT CCCCNTTCT TTCTTTTCTC TANTTTTTTC CNNGGNACGG GTTGNGGTNA	2400
ATNAANNCCC CTCCTTCGTC TATTCANCCC TTCCTATGNA CACTTCCTGN CCCCCTATCT	2460
CTCTATNTNC TNCTCTCTAT ATCTNNATCC CNTCTTCNCN TGCCNCTCCC TNGTNTTNA	2520
NCGGGTATTT NTNTTCTCC TCNTCTTCTT CCCCTNTNTA NCCNTNCTNC NNNCNNNCCC	2580

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 830 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "5H-1 (Example 10)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TGGGGGAGAG GACTGAAATA TTTCCACAGC CTTTTTATTG GTGGTGATGG TAGTGATGGT	60
TAGGATTCCT TCTTCTTTC TTTCTTCTT TCTTCTTTC TTTCTTTTTT TTTTTTTTTT	120
TTTTTTTTTT GAGACAGGGT TTCTCTGGGT ACTCCTGGAA CTCACTTTGT GGACCATGAA	180
TGACATGAAT ACTTCGATAT ATACATACAT ACAAAGACAC ATATTTTTTAA AAAGAGAATT	240
AGAGTAGAGC TGGGGCAATT GTGGAACACA CCTTTAACCT CAGGCAGATT TCTGCGTTCA	300
AGGTCACCTT GGATTACAAG GCAGCTAGGG CTACACAGAG AAACCATATC TCAAAAAAAA	360
GAAAAAATAA TGAAAGAAAG AAAGGAAGGA AGGAAGGAAG GAAGGAAGGA AGGAAGGAAG	420
AAAGGAAGGT AGGAAGAAAG GTATTTTCCT AAAAAAAAAA AAAAAAAAAA TTTATTCCGG	480
GCAGTGGTGG CAAATGCTTT TAATCCCACC ATTTGGGAAA GCAGAGGCAG ACAGATTAAA	540
TTTTCAAGGC CCACCTGGTC CTACACAGTG AATTCCAGGA ACACCTAGGT TTACCCANAA	600
AAAACCCCCC CTTGAAATAA AAAAAATAA ATTAAATAA TAAATTTAA AAATAAAACC	660
CGGGCGTTAA ACCCNCITTT ATCCCCCAC TTNGGAAGCA AAAGCCGGCN GATTTCTGAA	720
TTNAGGCCN CCCTGTCTAT GAATTANTTC CCNGAACACC CNAATTTTTC NAAAAACCCC	780
CCNTTCTTA AAAAANCCAA ATTATTATTN ATTAATTAAA TNAAATTACC	830

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 838 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "AN8T7 (Example 10)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGAGTCCAAC AATGGTTTCC ACTTGTCTGG CGGCCGCTCT AGAGTTTCCC ATAAGCTGGA	60
CTGAGAGATG GTGTGATTGC TGTGGGTGAC AAAGACAGAG GCACCTTTCA TCTCTACCCT	120
TCTCTTGTTT TGTTGTTTGT TTGAGACCGG TTCCCACTAT GTAGACCAGG CTGGAGGACA	180
GGGTCTCACT ATGTAGACCA GGCTGGCCTT GAACTCAAAG ACATCTGCCT GCCTCTGCCT	240
CCTGAGGGCT GGGATTAAAG GCGTGTGCTG CCACTGACAG CTTCTATCCT CCTGTCATCA	300
GTCCCGGCTC ACAGGGCCAG AAGATCTCTT CTATGCTTCC ACTATTTCCC CAATCCATTC	360
CCACGGCAGC CTCTCCATCT CCCTACCACC AAGACAGCAG CCTAGTGATA TAACAAAACCT	420
TTTATTCACA GGAAACCGGA AAACAAAATC ACAACCAATC ATTTCTATCT AGTCCCTGCC	480
CTAGCCCTCC CTCCAAGCCC CTACATATCC TCCATCTGAG GGGGATGCAT GCGTTGGGTG	540
GGAGCTGCCG GCATCCTTAT CCTGGTTCCT GGAGTAGNGA AGAGTGGTTC TTTTCAACGN	600
CTAGGGNNCT CCCCTCCAAG TTTGGACCTC TCTTCCCAGG NCTTCNCCCC TCCCTNACAG	660
GGNACAAAAA ACCAGGNACG GCACNACGCC AGGNAGGAAG GGA CTCTTGG NAATGTTGGG	720
CAGGACTTGT CCTCAGAATT CCNNGGAGGA ATCAAGGGCC TTGAATTCGG GAACCACTNC	780
CGAGGNCTTC ANCANGGCAN AGTTCAATTT TCCATCCCGG TTGGCCCANCTG GGCNG	838

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 180 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "CHINT (TABLE 5) "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TAGTGCCGGT CAAGGAACTG AACGTGCGAT TCCGGGACAG GCTACCCACT CCGATCCCAG	60
GAGAAGTTGT CATGGTGAGG GCCACCCTAG GTCTCTGCCC CTGCTGTGTC CCCCATCTTA	120
CCCATCCAGT AGGATCTAGA GGCTGTGCGC CCCTTGTTGA ATGCACAGAA GTCACAAGCG	180

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 175 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "HUMMDB (TABLE 5) "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CCTCACCTCC GCCCTGTTTC GTCCAGGTCC TCCGGGTCAG GCTACCCCCG TCGCCGCCAG	60
AGCGCGGGGG AGGGGAGAGC TTCCTTTGTC TCCTATGCCT CCTCCCCCA TCCCGGCTCT	120
CCTGCGGGCA AGCGCCGAGG GGACACCGGG GAGTACCCA CCTGAACCTC TGGGG	175

CLAIMS

What is claimed is:

- 5 1. A method of detecting cancerous cells in a patient by detecting alterations of PP2C α gene activity in a specimen isolated from the patient.
- 10 2. The method of claim 1 wherein the specimen is selected from the group consisting of tissue biopsies and bodily fluids.
- 15 3. The method of claim 1 further characterized by the alteration being a reduction in PP2C α gene activity compared to normal controls.
- 20 4. The method of claim 1 wherein said detecting steps is further defined as assaying the specimen for mRNA complementary to PP2C α DNA including polymorphisms thereof with an assay selected from the group consisting of *in situ* hybridization, Northern blotting and reverse transcriptase - polymerase chain reaction.
- 25 5. The method of claim 1 wherein said detecting step is further defined as assaying the specimen for a PP2C α gene product including polymorphisms and peptide fragments thereof with an assay selected from the group consisting immunohistochemical and immunocytochemical staining, ELISA, RIA, immunoblots, immunoprecipitation, Western blotting, functional assays and protein
30 truncation test.
- 35 6. The method of claim 5 wherein the specimen is bodily fluids selected from the group consisting of urine, blood, cerebrospinal fluid and saliva.

-81-

7. The method of claim 1 wherein the detecting of PP2C α gene activity in a specimen is by determining alterations in phosphorylation patterns of proteins affected by the PP2C α gene product.

5

8. A kit for detecting PP2C α activity as set forth in claim 4, said kit comprising:

a molecular probe complementary to genetic sequences of a mRNA for PP2C α including polymorphisms thereof and

10

detection means for detecting hybridization of said molecular probe and the mRNA thereby indicating the activity of the PP2C α gene.

15

9. A kit for detecting a gene product associated with PP2C gene activity as set forth in claim 5, said kit comprising:

an antibody which with high specificity recognizes markers selected from the group consisting of the PP2C α gene product including polymorphisms thereof and peptide fragments thereof, and

20

detection means for detecting the binding of the antibody thereby indicating the presence of the gene product.

25

10. A kit for detecting a gene product associated with PP2C gene activity as set forth in claim 5, said kit comprising:

an agent which mimics natural proteins which bind to the PP2C α gene product including polymorphisms thereof and peptide fragments thereof, and

30

detection means for detecting the binding of the agent thereby indicating the presence of the gene product.

35

11. A non-human transgenic mammal or cell line containing an expressible nucleic acid sequence for human PP2C α including polymorphisms thereof.

5 12. A non-human eucaryotic organism in which the equivalent genomic nucleic acid sequence for PP2C α is knocked-out.

10 13. A vector comprising an expression control sequence operatively linked to the nucleic acid sequence of PP2C α .

15 14. A host cell transformed with the vector of claim 13.

15 15. A vector comprising an antisense sequence of PP2C α .

20 16. An antibody which specifically binds to an epitope of a gene product of PP2C α including polymorphisms thereof which distinguishes the gene product of PP2C α from the gene product of PP2C β .

25 17. An antibody of claim 16 conjugated to a detectable moiety.

18. An antibody of claim 16 selected from the group consisting of monoclonal and polyclonal antibody.

30 19. A polyclonal antibody of claim 18 raised against recombinantly produced PP2C α .

35 20. A polyclonal antibody of claim 18 raised against the carboxy terminal peptide of pp2c α selected from the group consisting of NDDTDSASTD (SEQ ID No:1) and YKNDDTDSTSTDDMW (SEQ ID No:2).

21. A monoclonal antibody of claim 18 which does not cross-react with pp2c β and which is raised against peptides selected from the group consisting of recombinantly produced pp2c α , NDDTDSASTD (SEQ ID No:1)
5 and YKNDDTDSTSTDDMW (SEQ ID No:2).

22. A monoclonal antibody of claim 21 designated as 2A3.

10 23. An isolated and purified peptide selected from the group consisting of NDDTDSASTD (SEQ ID No:1), YKNDDTDSTSTDDMW (SEQ ID No:2) and PNKDNDGGA (SEQ ID No:3).

15 24. The peptide of claim 23 produced recombinantly.

25. A method of treating cancer including the steps of
20 a. determining the type of cancer and cells expressing the cancer,
b. preparing a vector which will specifically target the cancer cells including regulatory elements to control the expressibility of PP2C α , and
25 c. administering the vector to the patient.

26. The method as set forth in claim 25 wherein the vector includes an AAV modified sequence or part of the AAV sequence.
30

27. The method as set forth in claim 25 wherein the vector contains the CHINT sequences.

28. The method as set forth in claim 25 wherein
35 the vector includes the silencer region (SEQ ID No:13).

29. The method as set forth in claim 25 wherein the vector includes the mini-silencer region (SEQ ID No:14).

5 30. A method of treating cancer including the steps of

- a. determining the type of cancer and cells expressing the cancer,
- 10 b. preparing an antisense vector which will specifically target the cancer cells to control the expressibility of PP2C α , and
- c. administering the vector to the patient.

15 31. A pharmaceutical composition consisting of a vector and a pharmaceutically suitable carrier wherein the vector is selected from the group consisting of a vector which will specifically target the cancer cells and including regulatory elements to control the expressibility of PP2C α and an antisense vector which
20 will specifically target the cancer cells to control the expressibility of PP2C α .

25 32. A method of treating diseases due to aberrant phosphorylation due to alteration of expression of PP2C α including

- a. preparing an antisense vector which will specifically target cells expressing aberrant phosphorylation to control the expressibility of PP2C α , and
- 30 b. administering the vector to the patient.

35 33. A method of suppressing gene amplification by interrupting unscheduled interactions of DNA polymerase α primase with the gene product of PP2C α by preparing an antisense vector which will specifically target the

binding region of DNA polymerase α primase to the PP2C α gene product and delivering the vector to the cells.

5 34. A method for the activation of the gene product of PP2C α expressed on the surface of a cell to induce signal transduction.

10 35. The method of claim 34 wherein an antibody is used to bind to the gene product of PP2C α .

36. A method of detecting cancer in a patient by detecting altered PP2C β gene activity in a specimen isolated from the patient.

15 37. The method of claim 36 further characterized by detecting an increase in PP2C β activity.

20 38. The method of claim 36 wherein the detecting of PP2C β activity is by assaying the specimen for mRNA complementary to PP2C β DNA including polymorphisms thereof with an assay selected from the group consisting of *in situ* hybridization, Northern blotting and reverse transcriptase - polymerase chain reaction.

25 39. The method of claim 36 wherein the detecting of PP2C β activity is by assaying the specimen for a PP2C β gene product including polymorphisms thereof with an assay selected from the group consisting
30 immunohistochemical and immunocytochemical staining, ELISA, RIA, immunoblots, immunoprecipitation, Western blotting, functional assays and protein truncation test.

35 40. An antibody which specifically binds to an epitope of a gene product of PP2C β including

-86-

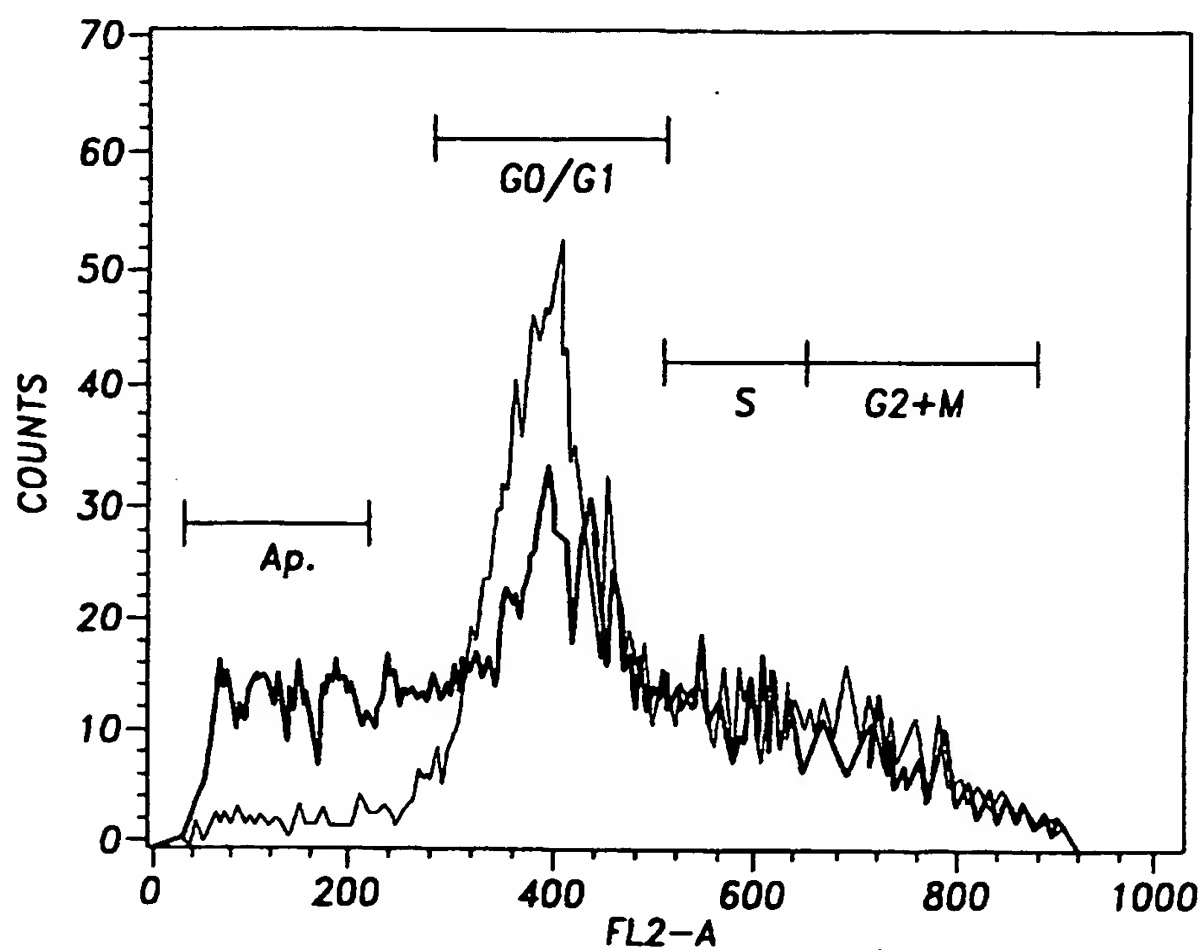
polymorphisms thereof which distinguishes the gene product of PP2C α from the gene product of PP2C β .

5 41. An antibody of claim 40 conjugated to a detectable moiety.

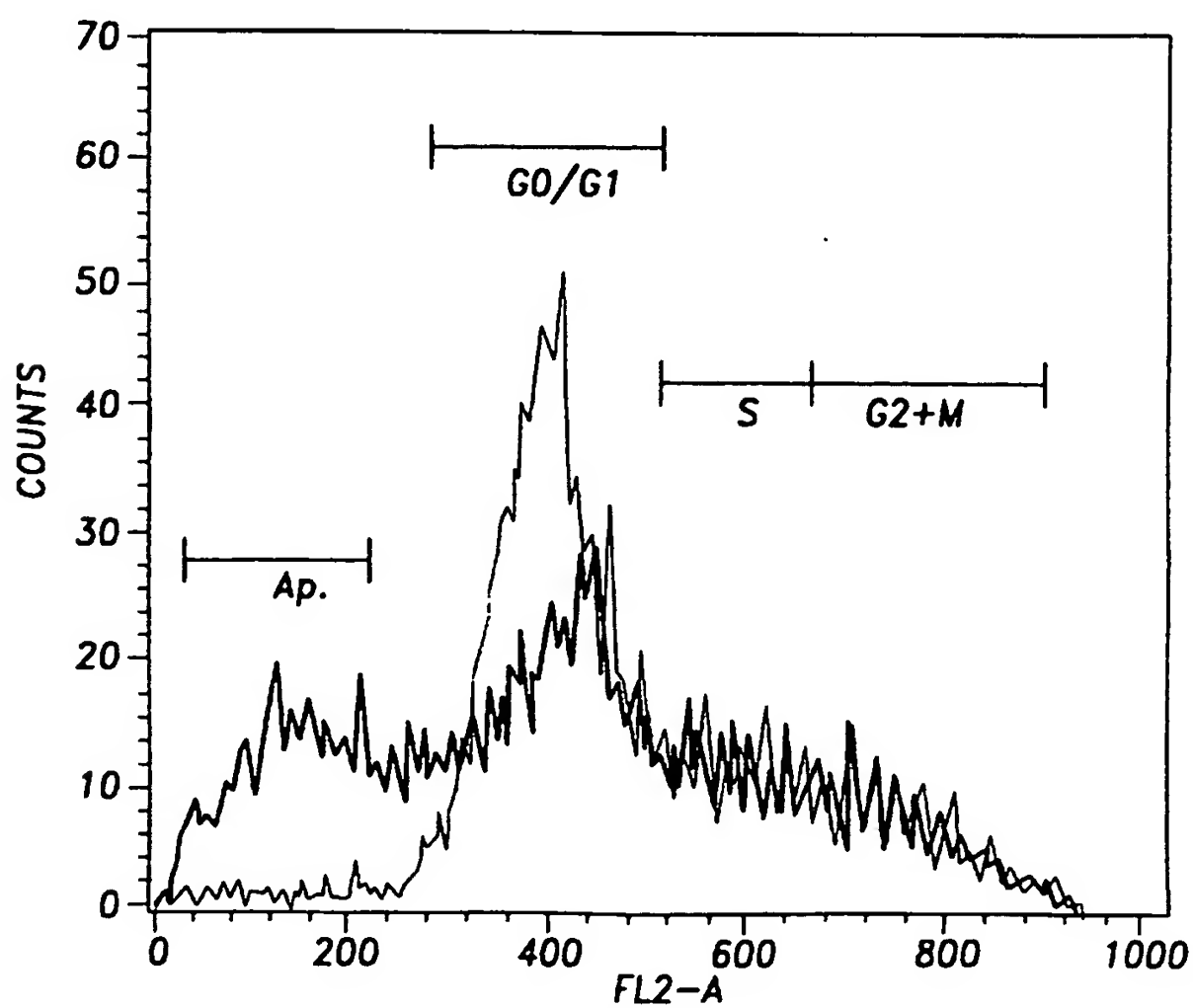
42. An antibody of claim 40 selected from the group consisting of monoclonal and polyclonal antibody.

10 43. A polyclonal antibody of claim 40 raised against recombinantly produced PP2C β .

15 44. A polyclonal antibody of claim 40 raised against the carboxy terminal peptide PNKDNDGGA (SEQ ID No:3).



KEY	NAME	PARAMETER	GATE	
—	SHU..005	FL2-A	NO GATE	Co60
—	SHU..008	FL2-A	NO GATE	913

Fig-1a

KEY	NAME	PARAMETER	GATE	
—	SHU..005	FL2-A	NO GATE	Co60
—	SHU..010	FL2-A	NO GATE	916

Fig-1b

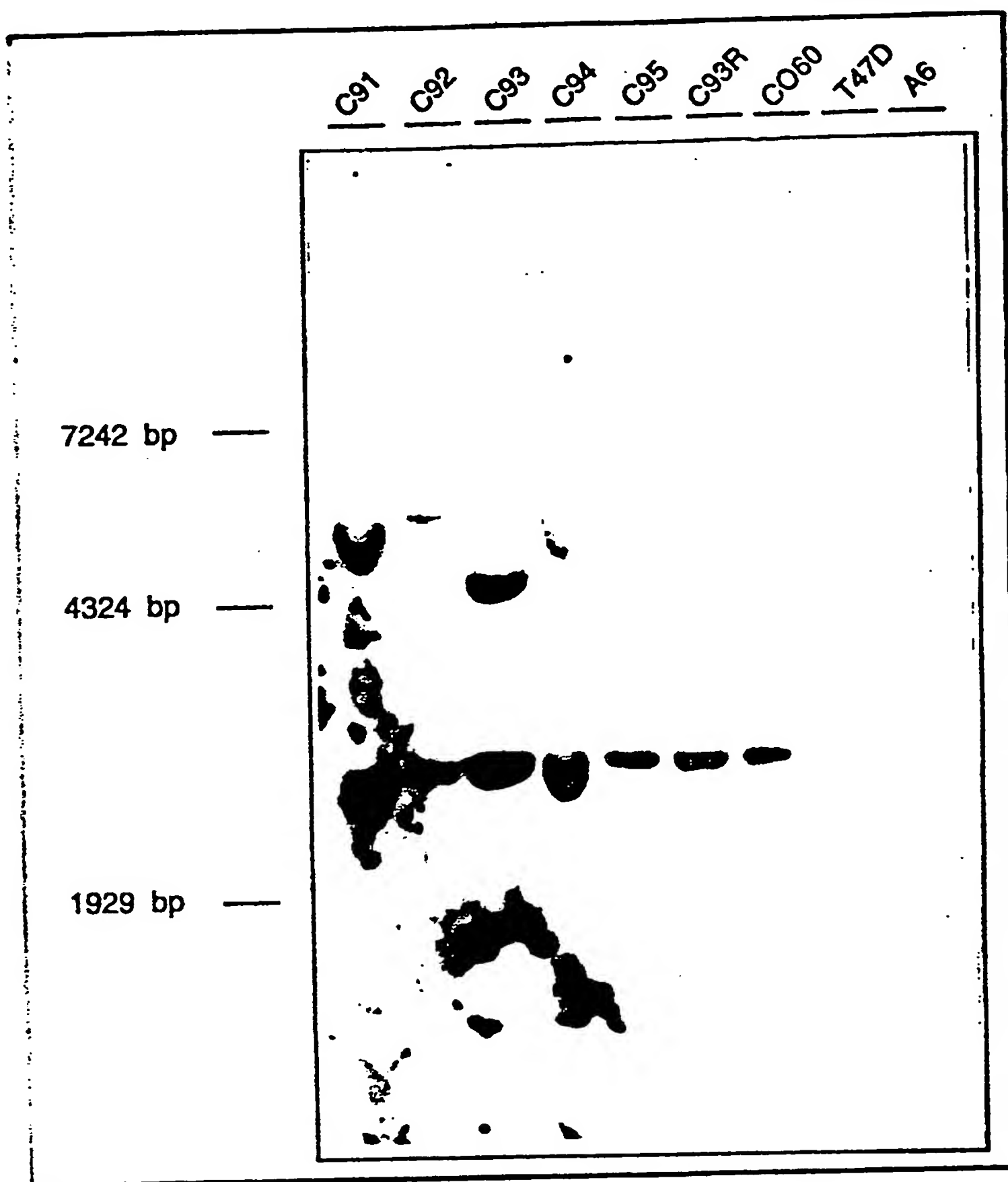
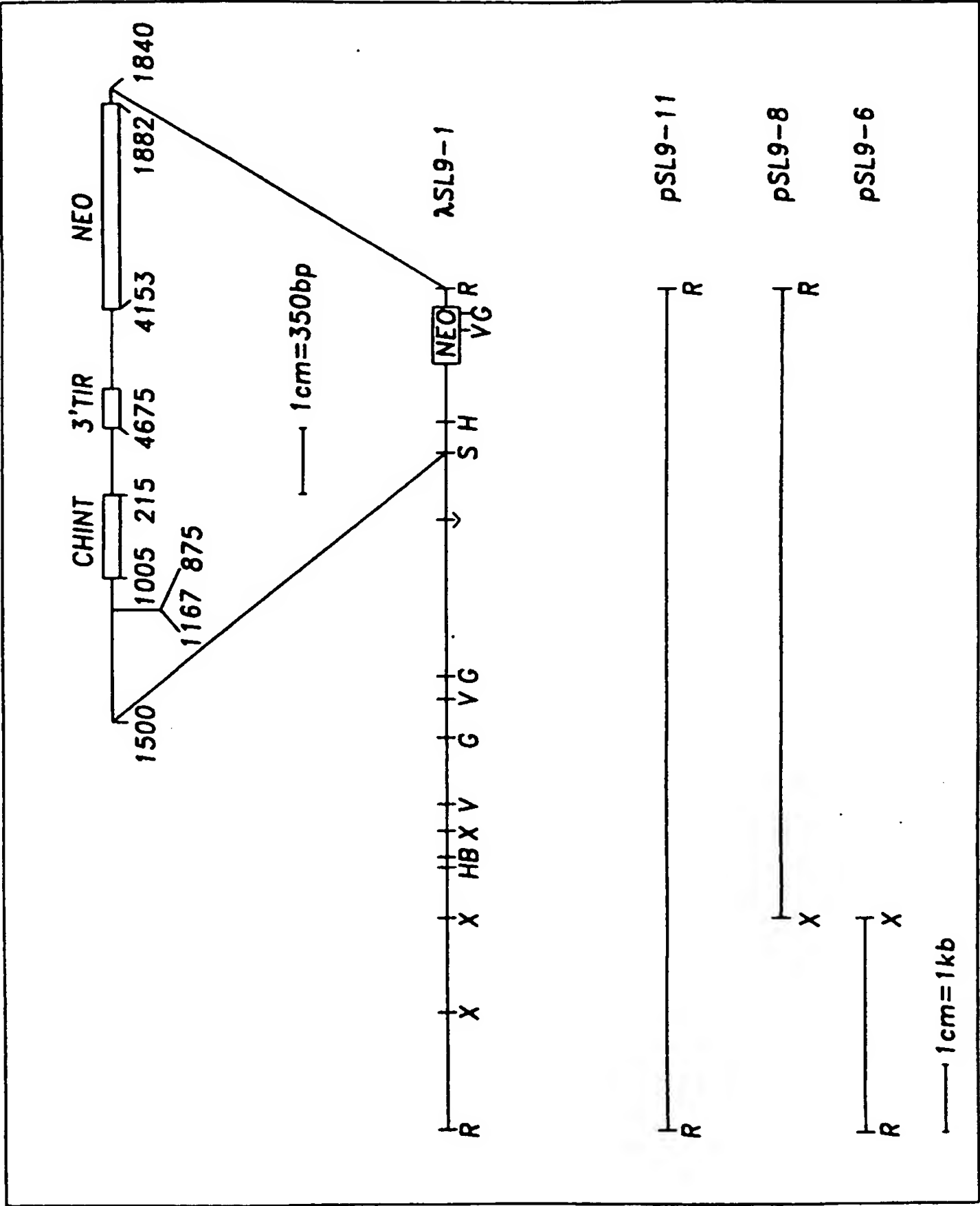
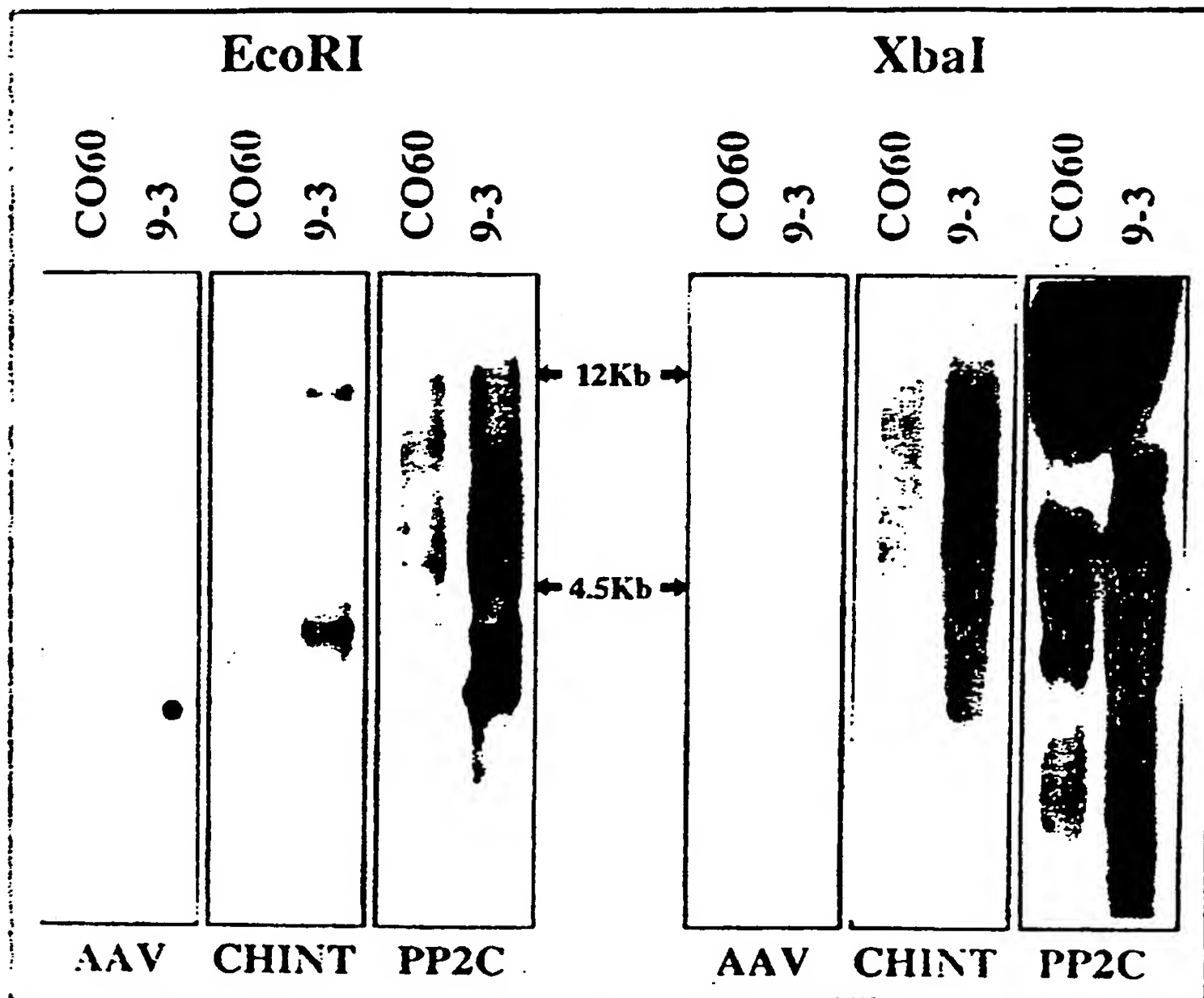


Fig-2

Fig-3



4/12

Fig-4a

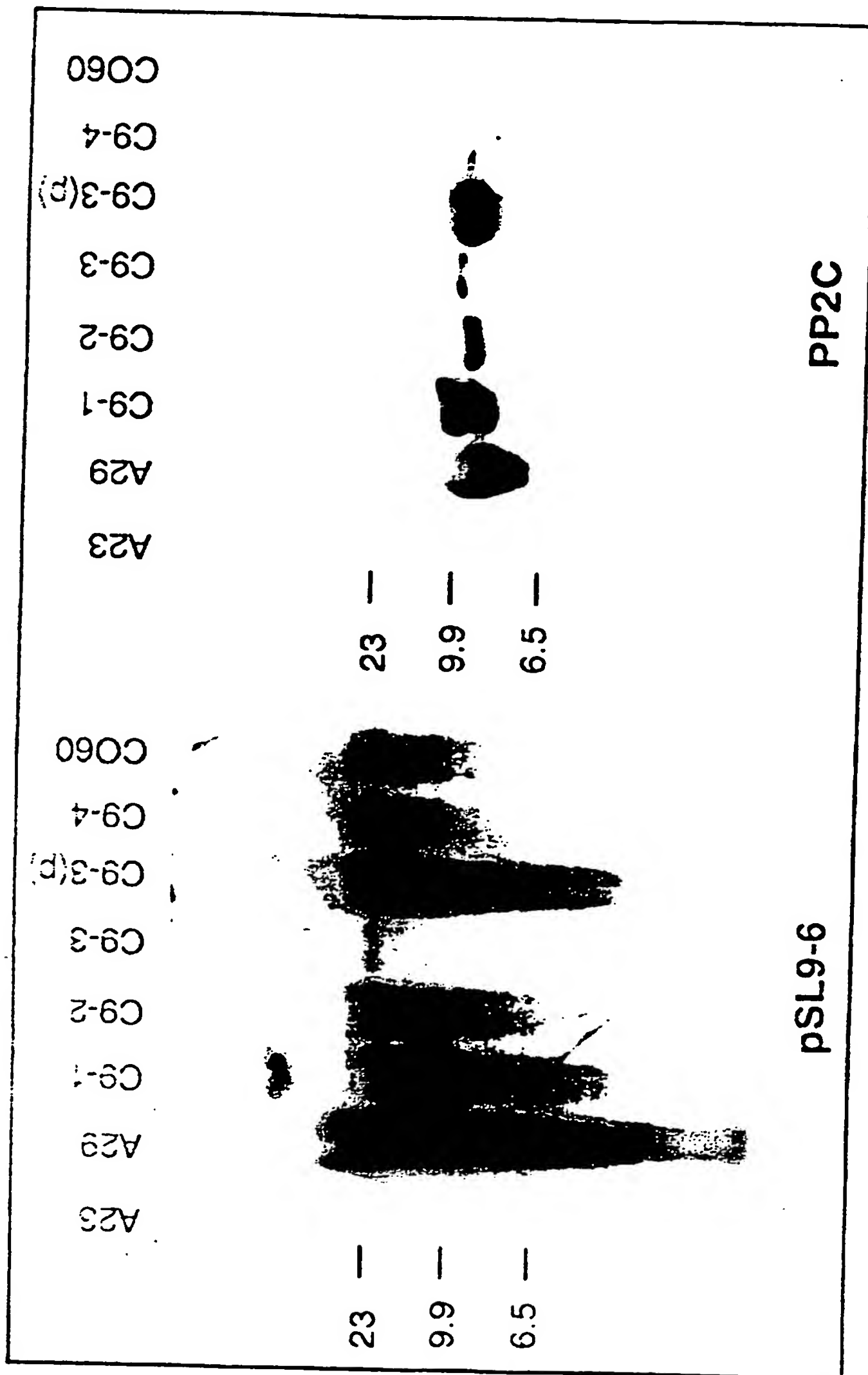


Fig-4b

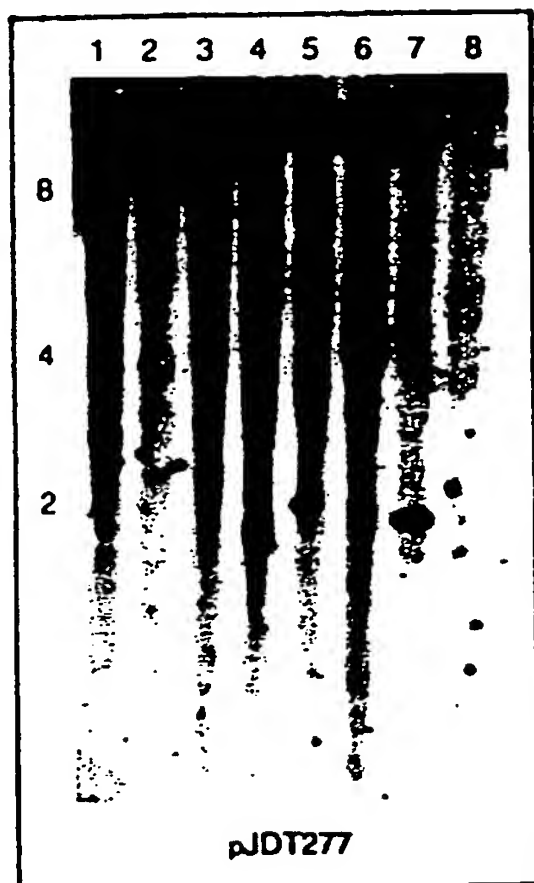


Fig-5a

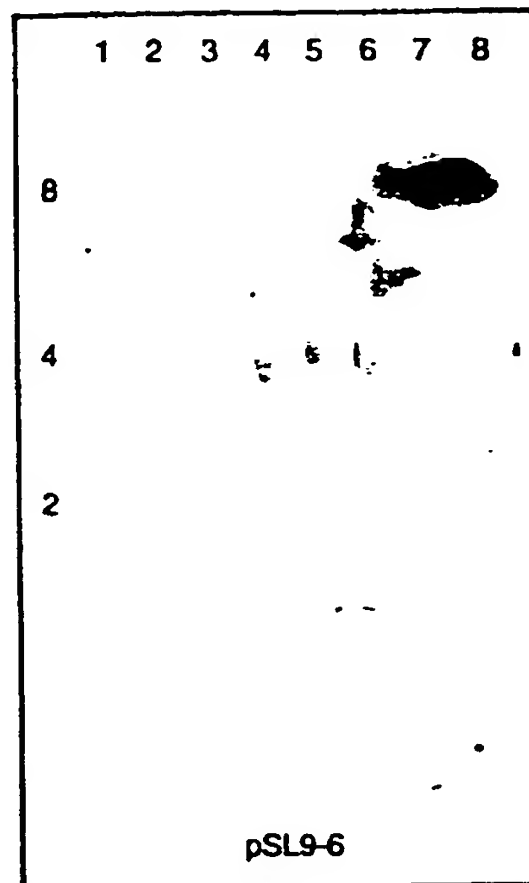


Fig-5b

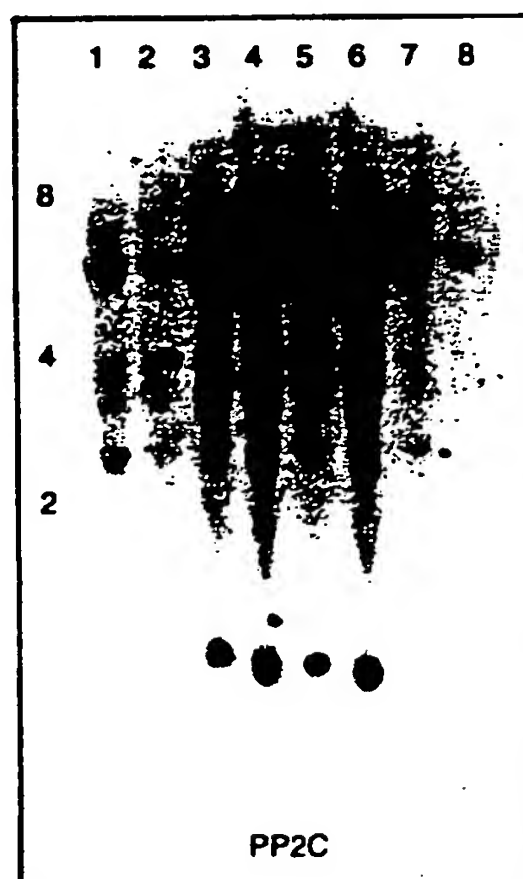


Fig-5c

6/12/1

Fig-6a

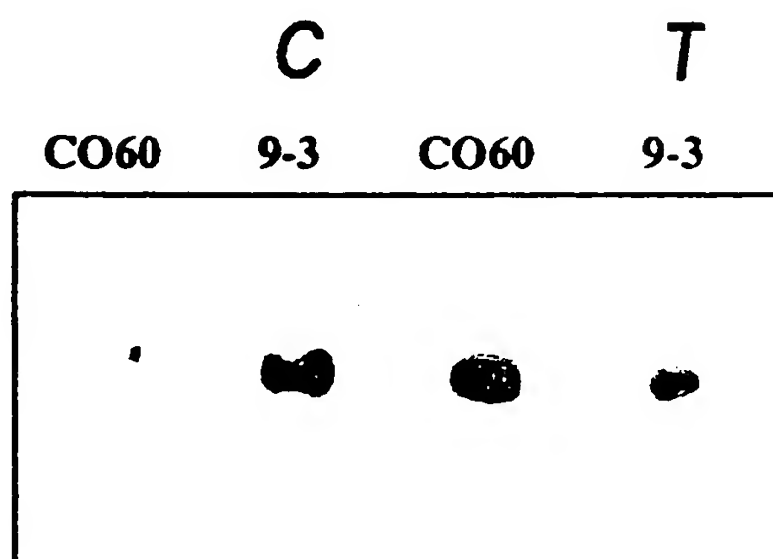
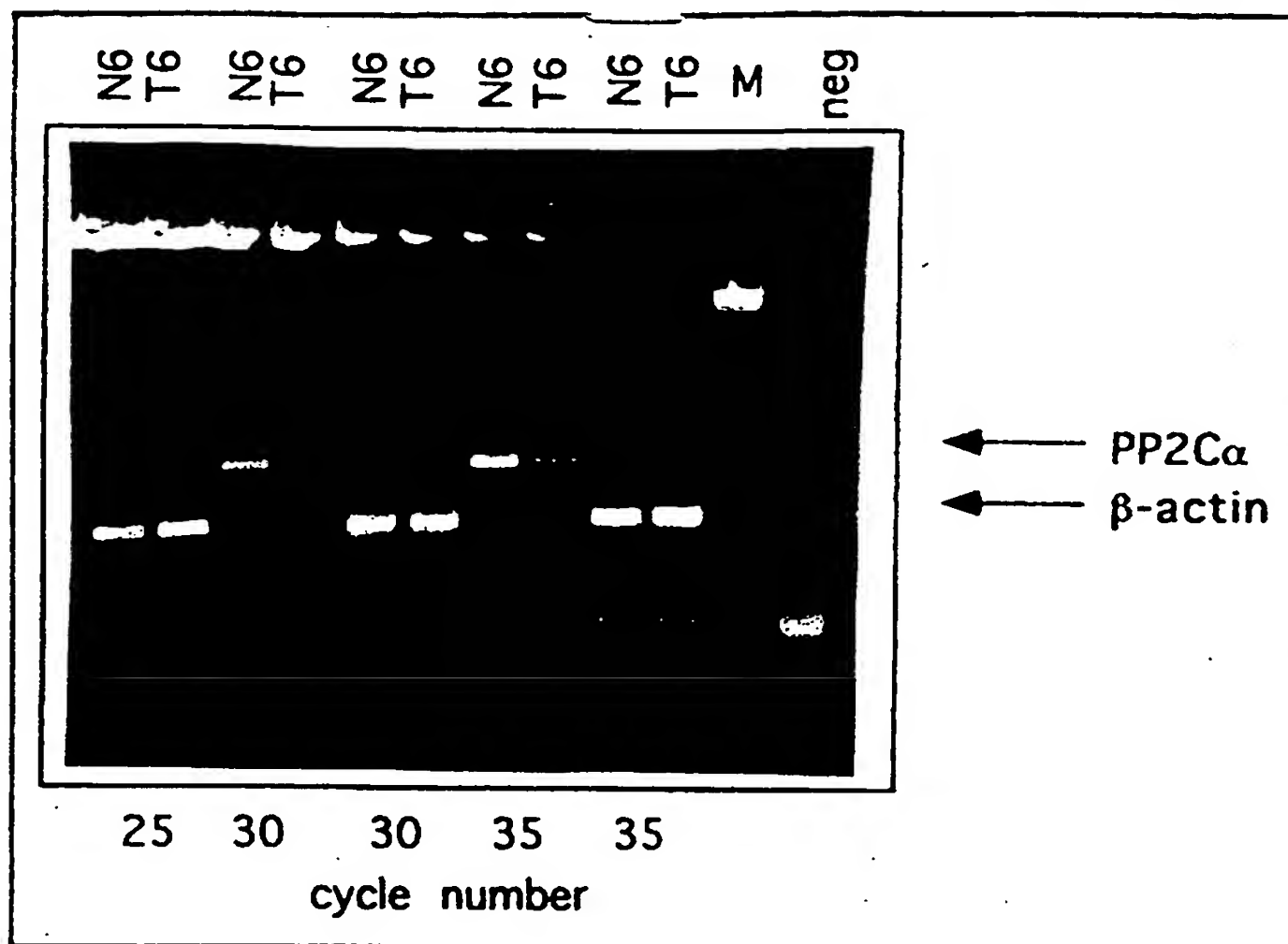
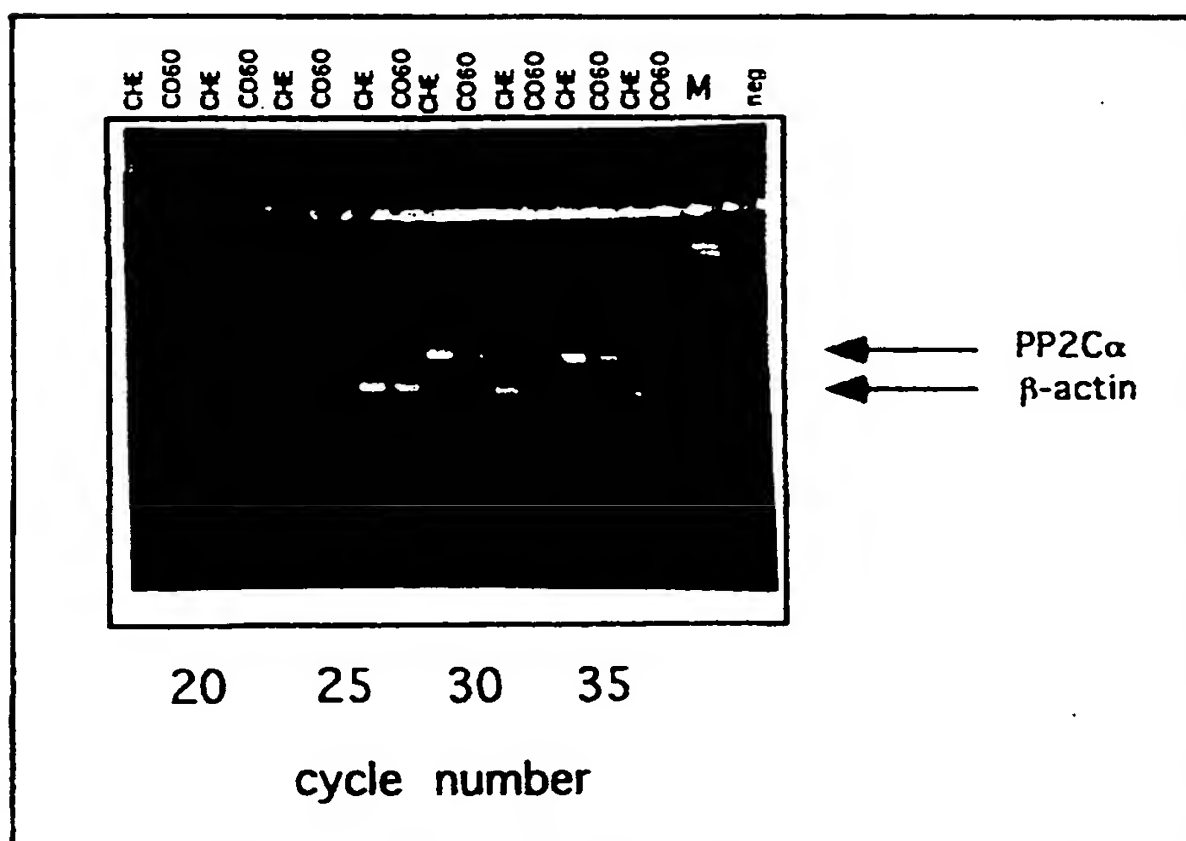
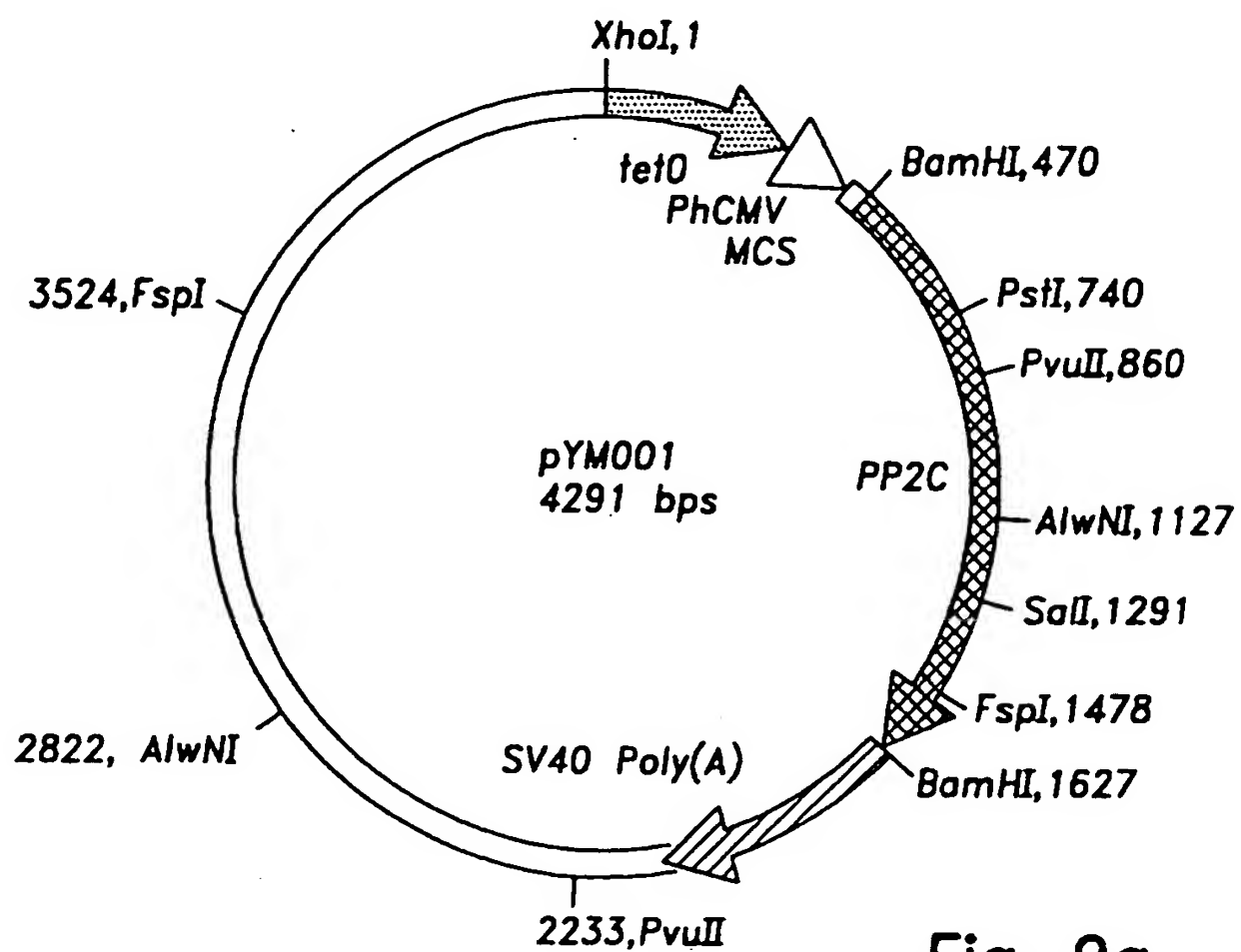
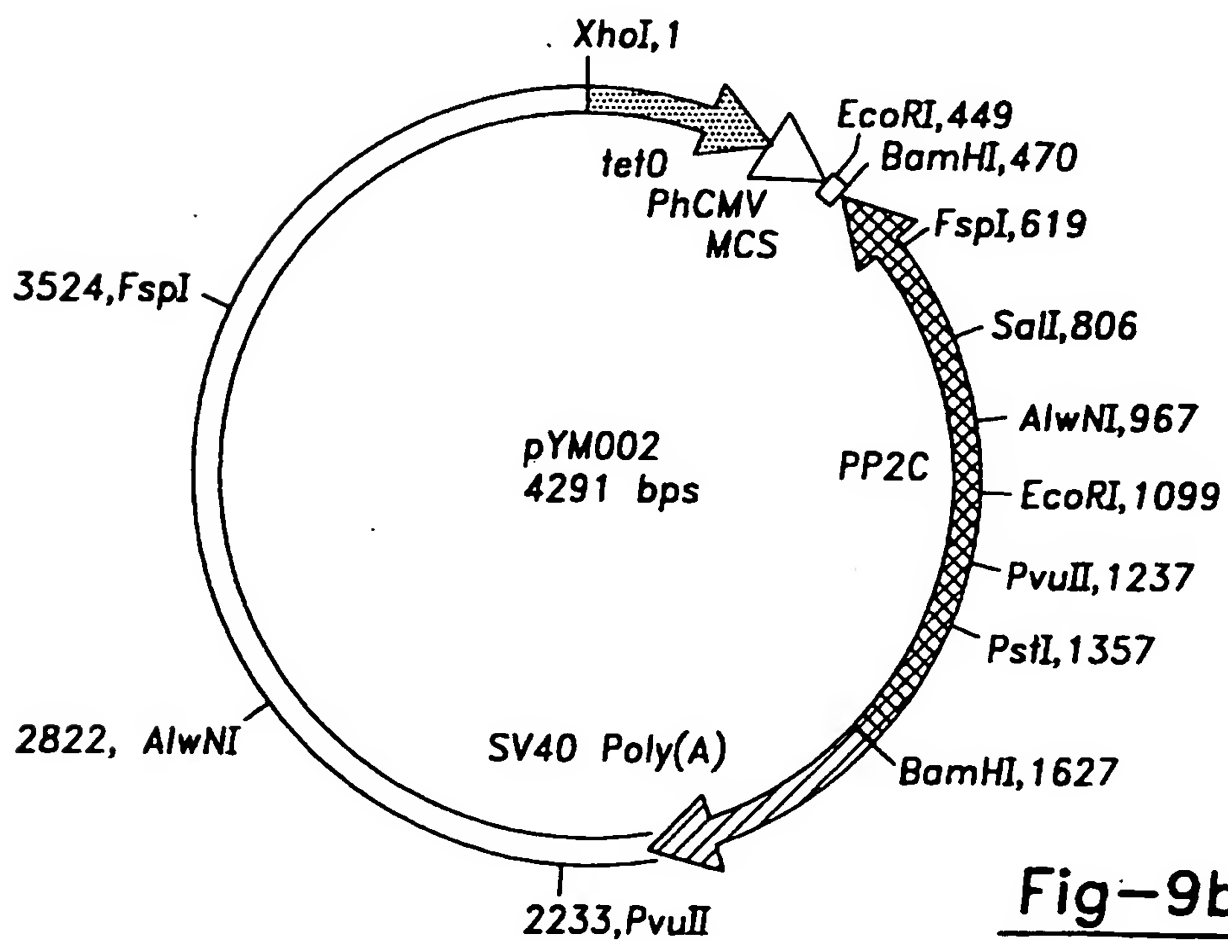


Fig-6b



Fig-7Fig-8

8/12

Fig-9aFig-9b

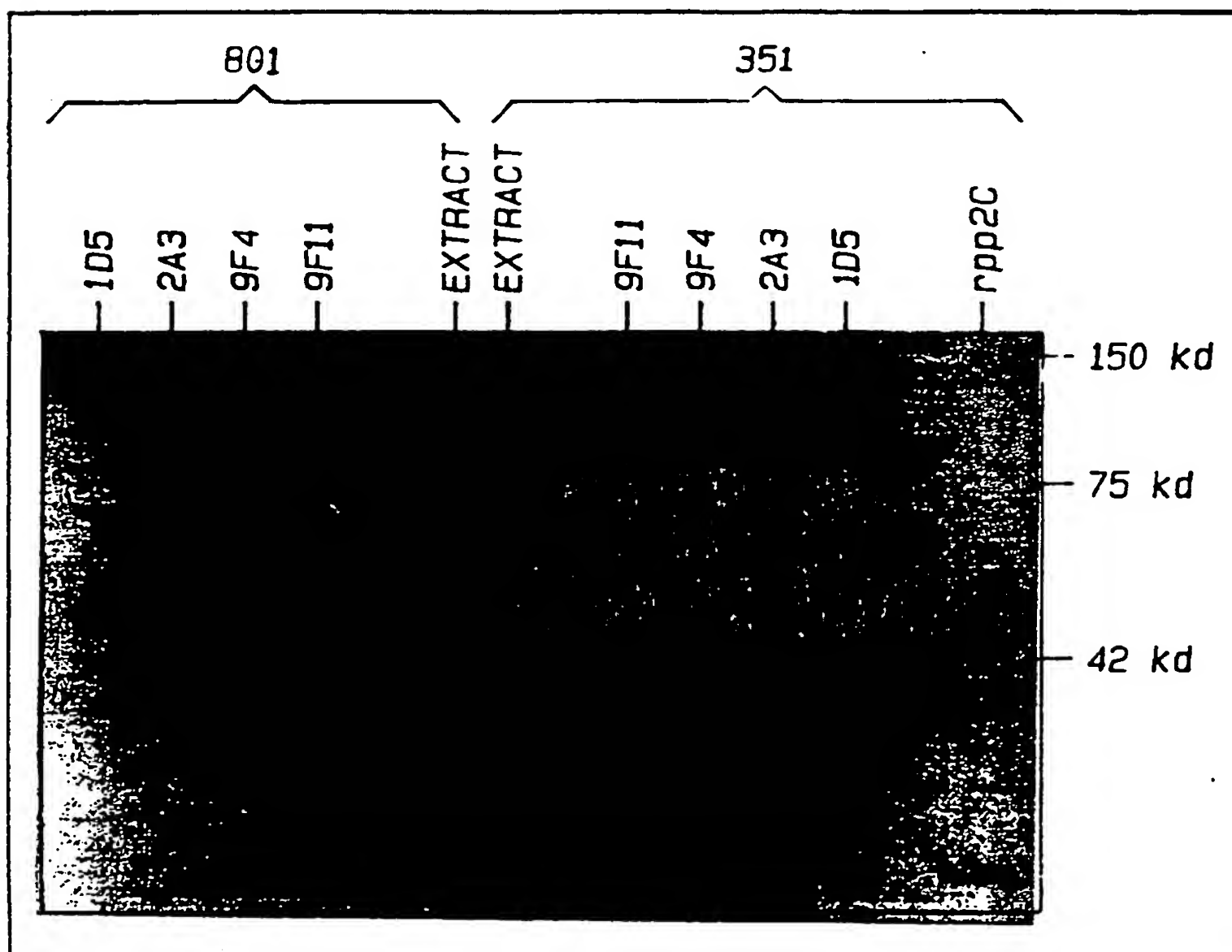
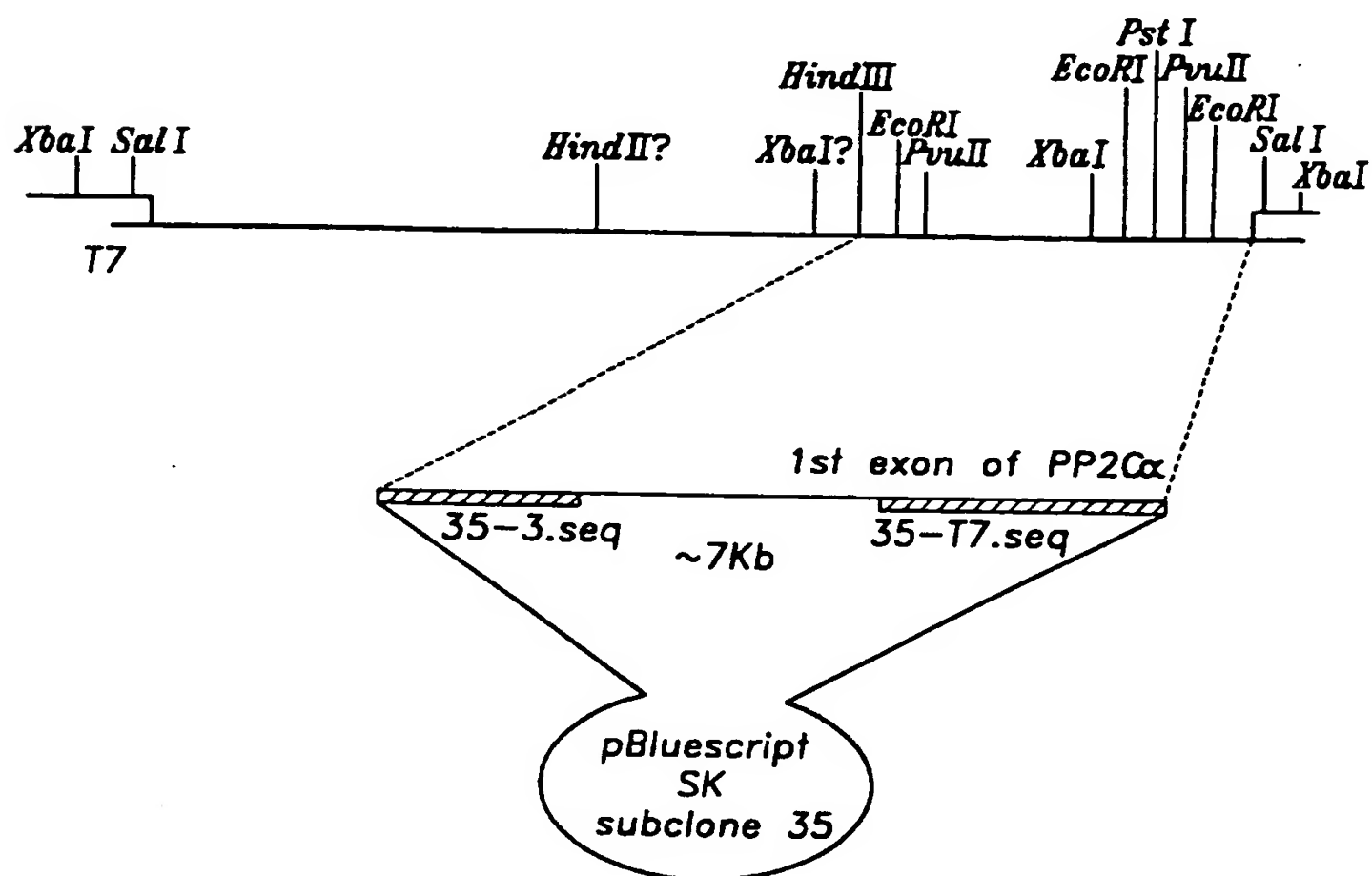


Fig-10

10/12

Fig-11

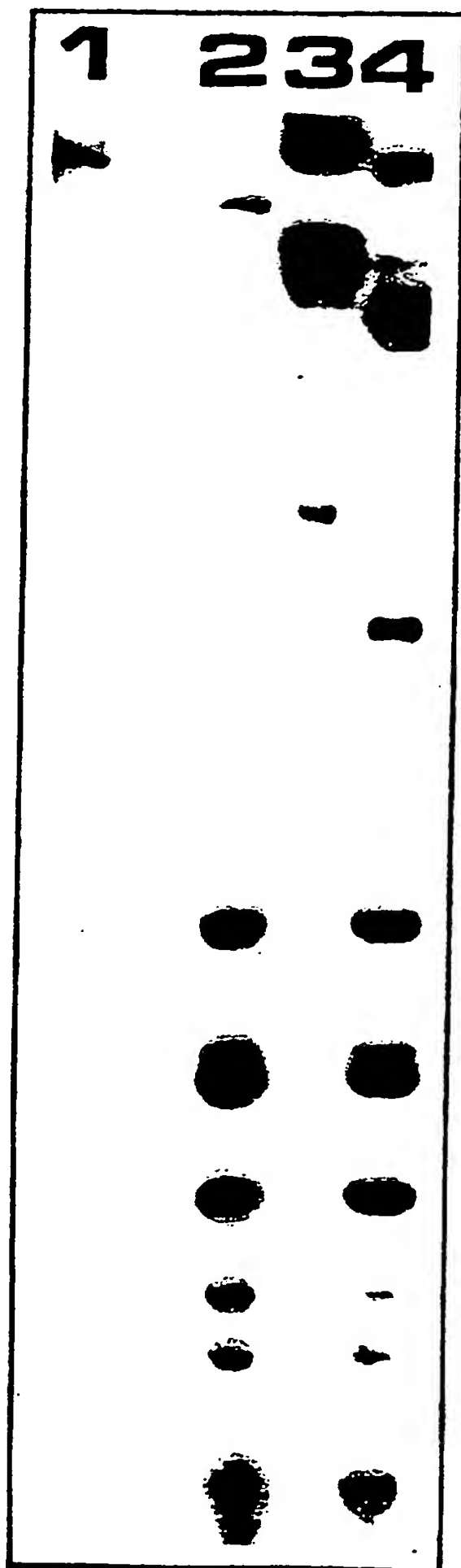


Fig-12

SUBSTITUTE SHEET (RULE 26)

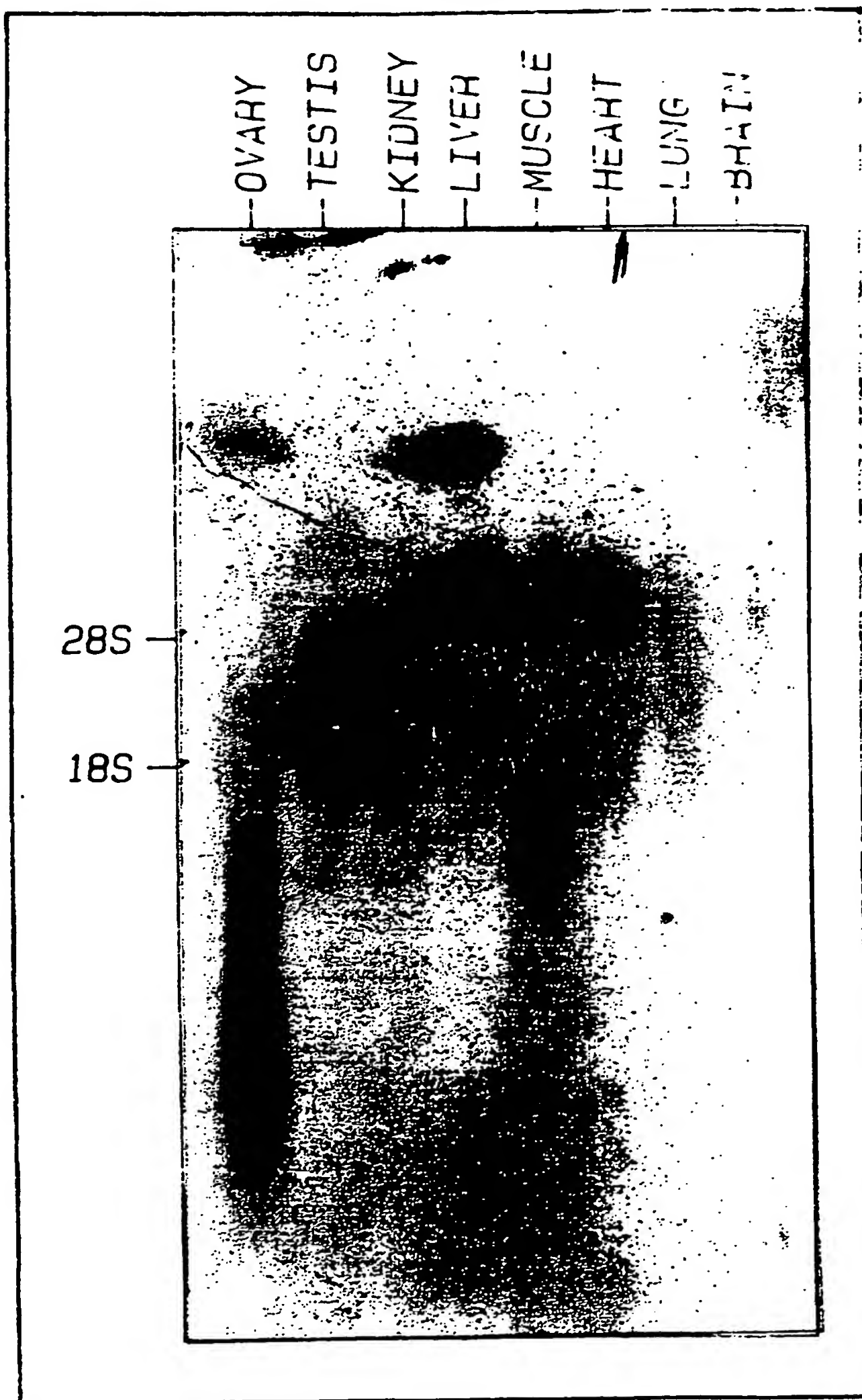


Fig-13